The Roles of Plant Sesquiterpenes in Defense against Biotic and Abiotic Stresses

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1. General Introduction*

Since the colonization of land by plants and the beginning of their sessile life style, plants have been constantly confronted with adverse biotic and abiotic stresses. However plants are not merely passive victims of these stresses; they have evolved various resistance strategies that encompass physical, developmental and chemical attributes. Plants and herbivorous insects, for instance, have coexisted for more than 350 million years and have developed a series of mutualistic and antagonistic interactions that shaped the basic biochemistries and population genetics of both organisms (Gatehouse, 2002). Based on this coexistence, Ehrlich & Raven (1964) coined the co-evolutionary theory which suggests that the evolution of plant defense chemicals and the stepwise evolutionary responses to these chemicals by insects have led to the species diversity of both groups. As a result of these defense and counter-defense processes, today more than 200,000 structurally diverse secondary metabolites of plant origin have been discovered (Mithöfer & Boland, 2012). Traditionally, secondary chemicals were once thought to be just metabolic wastes and detoxification products (Hartmann, 1996), however, in his seminal paper entitled "The Raison d'Etre of Secondary Plant Substances", Fraenkel (1959) presented the first evidence that most plant secondary chemicals had evolved to defend plants against insects and other natural enemies. Since secondary metabolism is usually dispensable for normal growth and development, its components can be continuously modified and adapted to the selection pressure of a continuously changing environment.

1.1 Diversity of plant defense strategies

Plant defense strategies can be generally categorized as constitutive or inducible (Chen, 2008). Constitutive defenses are always present irrespective of herbivore attack, while inducible defenses are activated following the trigger from enemy confrontation (Mithöfer & Boland, 2012). The effect of constitutive and inducible defenses on herbivores can be either direct or indirect.

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Direct defenses include plant traits that by themselves affect the susceptibility of host plants to herbivore attack (Kessler & Baldwin, 2002). On the other hand, indirect defenses include plant traits that by themselves do not affect the susceptibility of host plants, but serve as attractants to natural enemies of the attacking herbivores (Hägg et al., 2013). Direct defenses can be morphological barriers such as trichomes, lignification, thorns or resin production, or chemical compounds which can act as repellents, deterrents, antinutritive and antidigestive compounds (Gatehouse, 2002; Howe & Schaller, 2008).

Volatile organic compounds (VOCs) are one of the chemical-based defense arsenals of plants. VOCs are highly diverse group of over 1700 different compounds broadly classified into three major groups: terpenoids, phenylpropanoids/benzenoids, and fatty acid-derived C6-volatiles (Dudareva et al., 2004; 2006; Maffei, 2010). The majority of these volatiles are synthesized via a few basic biosynthetic pathways leading to one or a few key metabolites from which numerous enzymatic modifications such as hydroxylations, acetylation, and methylations create the diversity of emitted volatiles (Hartmann, 1996; Dudareva et al., 2004). Since the focus of this thesis is on volatile-based plant defenses, following I will present a brief overview of the ecological functions of VOCs in general.

1.2 Ecological functions of plant volatiles

Plants usually respond to herbivore attack by releasing a variety of plant volatiles. The composition and quantity of volatiles released may vary with the type of plant species attacked or the herbivore species attacking. In addition plant volatiles might be activated by exposur to few abiotic stress factors. Generally, these volatiles could function as toxins or deterrents against herbivores and pathogens, signals for plant-plant or plant-insect communications, and as a mechanism for abiotic stress resistance (Fig. 1.1).

1.2.1 Toxins and deterrents for direct defense

Several plant species may store volatiles in large pools found in specialized secretory structures such as glandular trichomes in Lamiaceae or resin ducts in conifers (Gershenzon et al., 2000). Such specialized structures minimize the risk of autotoxicity while maintaining high volatile concentrations in plant parts where defense is necessary

(Theis & Lerdau, 2003). Upon tissue disruption, the volatiles are released and function as a first line of direct defense against herbivores or microbial pathogens (Langenheim, 1994; McGarvey & Croteau, 1995). For instance, in conifers a complex mixture of terpenoids (termed oleoresin) composed of monoterpenes, sesquiterpenes, and diterpenes, is an important defense strategy against bark beetles and their associated fungal pathogens (Gershenzon & Croteau 1991; Langenheim 1994).

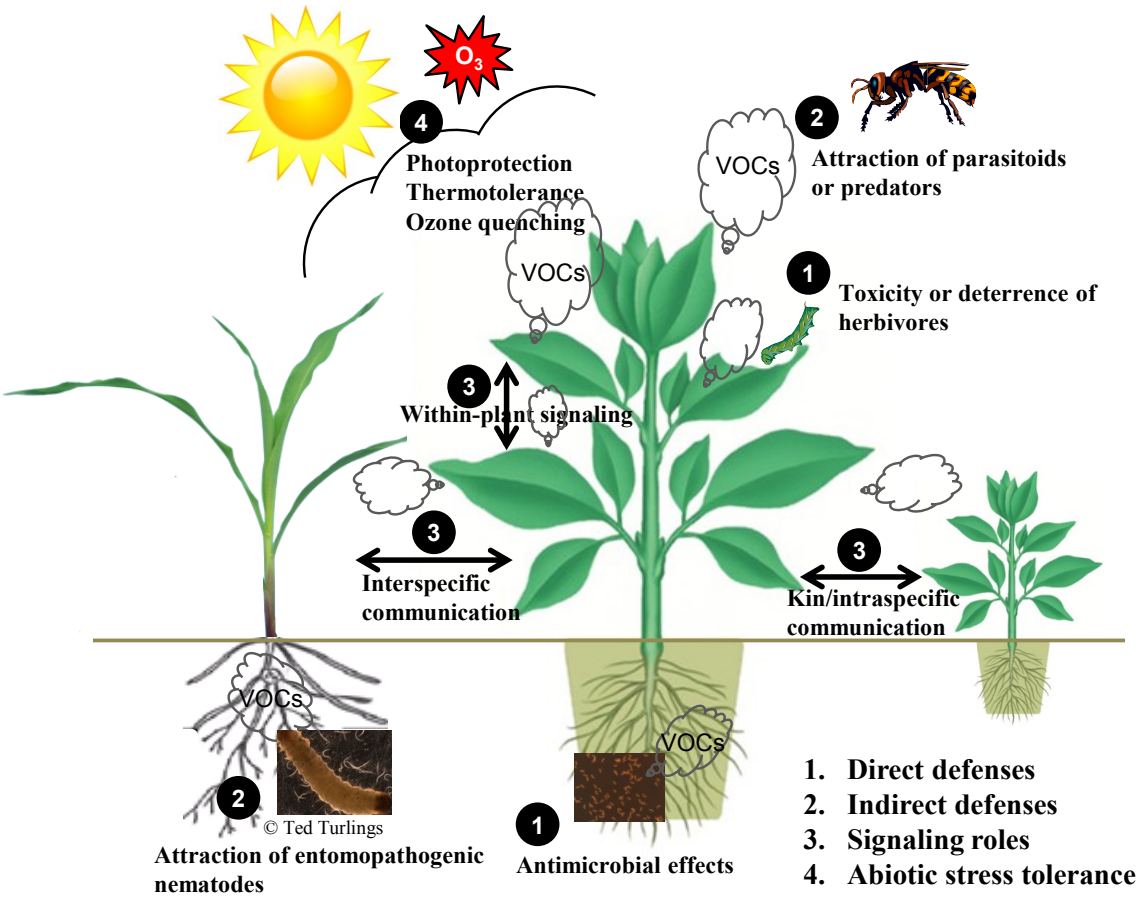

Fig. 1.1: A simplified overview of volatile-mediated ecological interactions. Plant volatiles may function as direct defenses against herbivores and pathogens **(1)** or indirectly protect plants by attracting natural enemies of the herbivores such as parasitoids or predators **(2)**. Volatiles also function as communication signals within and between plants of the same or different species to prime or elicit defenses **(3)**. Plant volatiles can also be recruited for protection against oxidative stresses generated by many abiotic stresses **(4)** such as high temperature, UV radiation and ozone pollution.

In plant species where volatiles are not stored, these compounds are released either constitutively or after herbivore damage in smaller quantities. Exposure to the volatiles may have negative impact on herbivore growth and development. Volatiles emitted by maize after damage by lepidopteran larvae, for instance, have a repellent effect on cereal aphids (Bernasconi et al., 1998). When plants are genetically engineered with

terpene metabolic genes, the plants strongly deterred herbivore feeding (Wang et al., 2001; Aharoni et al., 2003; Laothawornkitkul et al., 2008).

In addition to vegetative volatiles, floral volatiles can function as repellents and phytoalexins against herbivores and pathogens (Gershenzon & Dudareva, 2007; Huang et al., 2012). Other than terpenoids, green leaf volatiles which are commonly emitted shortly after herbivore damage were also shown to deter oviposition by lepidopteran herbivores (De Moraes et al., 2001; Kessler & Baldwin, 2001). Toxicity of green leaf volatiles towards aphids was demonstrated by experiments with transgenic potatoes in which the levels of these compounds were reduced by silencing the key biosynthetic enzyme, hydroperoxide lyase (Vancanneyt et al., 2001).

1.2.2 Signals for indirect plant defense

When attacked by herbivores, plants usually emit a blend of volatiles that can function as a 'top-down' control of herbivore population by attracting natural enemies of the attacking herbivores, a phenomenon termed indirect defense or 'plants crying for help' (Dicke, 2009). Since the volatile signals that orchestrate this phenomenon are mostly activated after herbivore damage, indirect defense is generally considered as cost-efficient defense strategy (Kessler & Baldwin, 2002; Dicke & Hilker, 2003). Although volatiles are indirect defensive traits expressed by many plants, other traits such as extrafloral nectar, food bodies, and structures used as refuge or nesting space (domatia) also function for indirect defense in some plant species (Heil, 2008). Among the best studied examples of this type of volatile-mediated interaction is the interaction between lima bean, herbivorous spider mites, and carnivorous mites (Dicke, 1999). Induction of volatiles to mediate indirect defense is not restricted only to herbivore attack; oviposition on plant surfaces by itself can trigger the release of volatile blends that serve for the attraction of egg parasitoids (Meiners & Hilker, 2000; Colazza et al., 2004).

The attraction of herbivore enemies by plants has obvious fitness benefits for the parasitoids and predators in guiding them to their hosts and prey. The fitness advantage for plants is also reported in few investigations. Under laboratory conditions, it has been shown that feeding by parasitized caterpillars reduced Arabidopsis fitness to a lesser extent than feeding by unparasitized caterpillars (van Loon et al., 2000). This result has also been obtained under field conditions using green leaf volatiles and terpenoids as

signals for egg predators on *Nicotiana attenuata* plants infested with three different herbivores where the degree of herbivory on the plants was reduced by as much as 90 percent (Kessler & Baldwin, 2001).

Since herbivore-induced plant volatiles are mixtures of compounds with high chemical diversity, the identities of the exact compounds responsible for signaling herbivore enemies are mostly unknown. However, recent progress in the isolation of genes encoding enzymes responsible for the formation of plant volatiles has allowed the use of genetic engineering as a tool to investigate the role of individual group of compounds in tritrophic interactions. For instance, Arabidopsis plants engineered with a single terpene biosynthetic gene resulted in plants which are more attractive to predatory mites and parasitoid wasps (Kappers et al., 2005; Schnee et al., 2006; Fontana et al., 2011). When terpene emission was compromised through down-regulation of certain biosynthetic genes, plants become less attractive to predatory bugs and mites (Halitschke et al., 2008; Mumm et al., 2008). In addition to terpenoids, green leaf volatiles have been also targeted for genetic manipulation to show their role in this regard. For instance, plants that are down-regulated for green leaf volatile emission have been found to be less attractive to parasitoid wasps and predatory bugs (Shiojiri et al., 2006; Halitschke et al., 2008).

While volatile compounds are usually studied in the context of above-ground plant interactions, some volatiles can also play a novel function as signaling molecules in below-ground plant interactions. The sesquiterpene (*E*)-β-caryophyllene, one of the best studied below ground signals, is released from maize roots in response to attack by the beetle *Diabrotica virgifera virgifera.* This volatile is used as a signal to attract predatory nematodes that safeguard maize roots from diabrotica attack (Rasman et al., 2005; Degenhardt et al., 2009; Robert et al., 2013).

1.2.3 Communication signals between and within plants

In addition to being signals for insect enemies, induced volatiles can also function as signals to convey information about future risk of herbivore attack to neighboring plants or unattcked part of their own. Since the first evidence of volatile-mediated plant communication (Rhoades, 1983; Baldwin & Schultz, 1983), a number of follow-up studies have confirmed the fact that volatiles from herbivore-damaged plants induce the

production of anti-herbivore defenses in many species (Arimura et al., 2000; Engelberth et al., 2004). Sometimes volatiles do not directly induce defenses, but act by priming the plant to respond more rapidly and intensively against subsequent enemy attack (Zimmerli et al., 2000; Conrath et al., 2006). In a primed state, plants partially turn on the machinery for defense-related processes without investing much in the formation of actual defense metabolites until the onset of subsequent attack (Conrath et al., 2006; Frost et al., 2008a). Hence, the fitness costs of priming are substantially lower than those of the directly induced defenses (van Hulten et al., 2006; Conrath et al., 2006).

When attack commences by herbivore enemies, primed plants activate either direct defenses that make them resistant to subsequent herbivore attack or indirect defenses that involve the recruitment of carnivorous arthropods as 'body guards'. These activations result from an increase in the accumulation of signaling molecules from the octadecanoid pathway that subsequently regulate the induction of antiherbivore defenses (Engelberth et al., 2004; Frost et al., 2008). Activation of plant secondary metabolites, increased transcription of defense genes, and enhanced protease activities are all commonly observed phenomenon in volatile-exposed plants (Arimura et al., 2000; Kessler et al., 2006; Ton et al., 2007; Peng et al., 2011; Hirao et al., 2012). In addition, primed plants show enhanced emission of other herbivore-induced volatiles (Engelberth et al., 2004; Frost et al., 2007, 2008, Ton et al., 2007; Rodriguez-Saona et al., 2009; Muroi et al., 2011; Li et al., 2012) or the accumulation of extrafloral nectar (EFN) secretion (Heil & Kost, 2006; Heil & Silva-Bueno, 2007) that can be an attractant to natural enemies of the herbivores.

Current research findings on volatile-mediated, within-plant signaling have opened a new era in the investigation of plant-plant communication, resolving many issues that were not explained from the context of between-plant signaling. Within-plant signaling by volatiles was first demonstrated in hybrid poplar leaves primed by volatiles from adjacent herbivore-damaged leaves that had no vascular connections to the primed leaf (Frost et al., 2007). The ability of a damaged part of a plant to rapidly signal the likelihood of future herbivory to adjacent plant parts is clearly beneficial. A similar within-plant volatile signaling has been demonstrated in blueberry plants when they are exposed to induced volatiles emitted from conspecific branches (Rodriguez-Saona et al., 2009). Volatile-mediated plant communication is currently being investigated further in the context of kin relationship, focusing on genetic as well as physical relationships

between communication partners. For instance, sagebrush plants in the field that received volatile cues from experimentally clipped close relatives were better protected from herbivore attack than those that received cues from clipped neighbors that were more distantly related (Karban & Shiojiri, 2009; Karban et al., 2013).

In addition to priming, exposure to volatile compounds may lead to a direct elicitation of defenses. For instance, exposure of Arabidopsis leaves to green leaf volatiles and monoterpenes has been shown to activate transcriptional responses and induce chemical and morphological defenses (Kishimoto et al., 2005, 2006; Godard et al., 2008). A direct volatile elicitation of defense metabolites such as extrafloral nectar secretion in lima bean and terpenoid volatile emission in maize has been also reported (Ruther & Fürstenau, 2005; Kost & Heil, 2006; Heil & Silva-Bueno, 2007).

Despite a large number of laboratory and field experiments showing volatile-mediated priming or defense elicitation in exposed plants, the mode of volatile perception and early molecular events that orchestrate the downstream activation of defenses are still poorly understood. However, investigations on Arabidopsis and tomato plants that were exposed to green leaf or terpenoid volatiles suggested the involvement of plasma membrane depolarization and alteration in cytosolic calcium levels as early events to amplify the volatile signal (Asai et al., 2009; Zebelo et al., 2012). Other recent investigations suggest the possible involvement of epigenetic factors such as DNA methylation as the molecular basis for the perception of volatile signals during priming (Ali et al., 2013). The authors found the expression of a Bowman-Birk type of trypsin inhibitor gene increased in maize plants that were exposed to conspecific herbivore-induced volatiles. Interestingly, a suite of methylation sites were found to be demethylated in the promoter region of this gene following volatile treatment.

1.2.4 Role in abiotic stress tolerance

The evolution of plant defense metabolites is usually considered to be tightly associated with defenses against herbivores and other biotic stresses. However, a few defense metabolites have been also proposed to have evolved primarily for abiotic stress tolerance (Holopainen, 2004). This assumption is based on the fact that when plants moved from aquatic to terrestrial life, the first stress they encountered was abiotic stress due to high levels of oxygen, which was non-existent in the oxygen-deprived aquatic

environment (Vickers et al., 2009a). Studies on abiotic stress tolerance mechanisms in plants have usually focused on nonvolatile compounds; however, a few key findings also suggest that plant volatiles may play a significant role in protection against oxidative and other abiotic stresses. Terpenoid volatiles are common examples considered in this regard as primitive traits evolved to cope with multiple abiotic stresses (Affek & Yakir, 2002).

For instance, when isoprene production is inhibited by fosmidomycin, plants accumulate more damaging reactive oxygen species and membrane degradation products under high temperature stress (Velikova et al., 2005; 2006). With exogenous isoprene fumigation the observed physiological disorders can be eliminated (Sharkey et al., 2001). A role for isoprene in thermotolerance has been also shown in transgenic tobacco plants created to emit isoprene (Vickers et al., 2009b). Like isoprene, monoterpenes are also described to have a role in heat stress tolerance (Loreto et al., 1998). For instance, blocking monoterpene emission in *Quercus ilex* (L.) leaves by feeding with fosmidomycin resulted in the disruption of the photosynthetic apparatus under heat stress; however, this effect was restored by exogenous fumigation of the plants with monoterpenes (Copolovici et al., 2005).

Although the mechanism by which isoprene or monoterpenes protect plants from heat stress is not known, it has been proposed that these volatiles may enhance membrane integrity and stability during high temperature stress (Sharkey & Singsaas, 1995). This assumption was recently supported by an *in vitro* experiment investigating the interaction of isoprene with a model phospholipid membrane under high temperature exposure. The results of this experiment indicated that isoprene partitions preferentially in the center of the membrane stabilizing lipid membrane and reducing the likelihood of a phospholipid membrane undergoing heat induced phase transition (Siwko et al., 2007).

In addition to involvement in thermotolerance, volatile isoprenoids are also able to protect plants from stresses caused by ozone (O_3) exposure. When isoprene or monoterpene production is inhibited by fosmidomycin treatment in *Phragmites australis* and *Quercus ilex* plants respectively, ozone exposure highly compromised their photosynthetic capacity (Loreto & Velikova, 2001; Loreto et al., 2004). These plants accumulated higher levels of damaging reactive oxygen species such as hydrogen peroxide (H_2O_2) and membrane peroxidation product malonyldialdehyde (MDA). By genetically transforming tobacco plants with isoprene synthase genes, the plants were

able to emit isoprene like the naturally emitting plants. When these plants were exposed to ozone, they accumulated less reactive oxygen species, developed reduced foliar damage, and increased their photosynthetic efficiency compared with non-emitting controls (Vickers et al., 2009). In isoprene emitting poplar, isoprene was suggested to effectively stabilize thylakoid membranes during ozone exposure; a similar effect was also observed when non-emitting tobacco and birch plants were exogenously fumigated with isoprene (Loreto et al., 2001).

Although the mechanism of isoprenoid protection of plants against oxidative stress caused by ozone still remains unclear, it has been proposed that isoprenoid volatiles may have a direct ozone quenching property at membrane level before the pollutant is actually taken into a plant cell (Loreto et al., 2001; Jardine et al., 2011). Volatile isoprenoids can also quench reactive oxygen species generated internally under several abiotic stress conditions. For instance, exogenous fumigation of isoprene has been shown to quench endogenous singlet oxygen species generated when plants were treated with Rose Bengal (RB) (Affek & Yakir, 2002). A direct interaction of singlet oxygen ($^1O_2$) generated by RB with isoprene was also studied in isoprene-emitting *Phragmites australis* plants (Velikova et al., 2004). When isoprene production was inhibited by fosmidomycin treatment and subsequently leaves were fed with RB, a dramatic decrease in photosynthetic performance and increase in H_2O_2 and MDA levels were measured. Since the topic of this thesis is on plant defenses with terpenoid volatiles, a brief overview of the biosynthetic mechanism of terpenes will be presented in the following section.

1.3 Biosynthesis of terpenoids in plants

The largest and most diverse group of plant volatiles belongs to the terpenoid compounds. Although structurally diverse, terpenoids share a common biosynthetic route and are composed of the basic 5-carbon building blocks (Pichersky et al., 2006): isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). In plants, two separate metabolic pathways are responsible for the biosynthesis of these building blocks (Fig. 1.2): the cytosolic mevalonic acid (MVA) pathway and the plastidic 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Dudareva et al., 2013). The two pathways can be linked through exchanges of metabolic precursors across the plastid membrane indicating that both pathways are not metabolically autonomous (Laule et al., 2003; Hemmerlin et al., 2003). In both compartments, IPP and DMAPP are used by

prenyl transferases in condensation reactions to generate larger prenyl diphosphates, such as the monoterpene precursor geranyl diphosphate (GPP), the sesquiterpene precursor farnesyl diphosphate (FPP) and the diterpene precursor geranylgeranyl diphosphate (GGPP). The terpene synthases (TPS) are the final enzymes to catalyze the formation of the primary terpene skeletons from the prenyl diphosphates (Aharoni et al., 2006). It is generally assumed that the final steps in the formation of hemiterpenes, monoterpenes, diterpenes and tetraterpenes occur in the plastids; whereas, the formation of sesquiterpenes and triterpenes takes place in the cytosol (Yu & Utsumi, 2009). Further enzymatic modifications such as hydroxylations, dehydrogenation, reduction, glycosylation and methylation add up to the diversity of terpenoids (Dudareva et al., 2004).

Terpenoid composition is not only markedly different at the metabolic level, but it is highly diverse from one species to another. Even within a single species, terpenoids often exhibit pronounced qualitative and quantitative variation among different varieties or ecotypes (Köllner et al., 2004a&b; Degen et al., 2004; Tholl et al., 2005). For instance, maize can synthesize and emit over 100 different terpenoid volatiles dominated by the sesquiterpene hydrocarbons, but the distribution is highly variable both quantitatively and qualitatively among different maize varieties (Turlings et al., 1998; Gouinguene et al., 2001; Köllner et al., 2004a&b).

Most maize sesquiterpenes are formed by five multiproduct terpene synthases which are expressed differentially throughout the plant (Köllner et al., 2004a; Schnee et al., 2006; Köllner et al., 2008). The herbivore-induced terpene synthase TPS10 produces sesquiterpene blends dominated by *(E)*-β-farnesene and *(E)*-α-bergamotene in young seedlings (Schnee et al., 2006). At a later developmental stage, maize emits volatile mixtures from its husks dominated by olefinic sesquithujene- and bisabolene-type sesquiterpenes produced from two closely related sesquiterpene synthases, TPS4 and TPS5 (Köllner et al., 2004a). The terpene synthases TPS7 and TPS8 are responsible for the formation of the constitutively emitted sesquiterpenes dominated by germacrene D and δ-cadinene (Fontana, PhD Dissertation). Finally, *(E)*-β-caryophyllene which is emitted by leaves and roots in response to herbivore attack is synthesized by TPS23 (Köllner et al., 2008). The main goal of my thesis was to characterize the functions of maize sesquiterpenes against biotic and abiotic stresses. Most of the investigations in this

thesis were carried out on Arabidopsis plants genetically transformed with the respective maize terpene synthases to produce and emit sesquiterpenes.

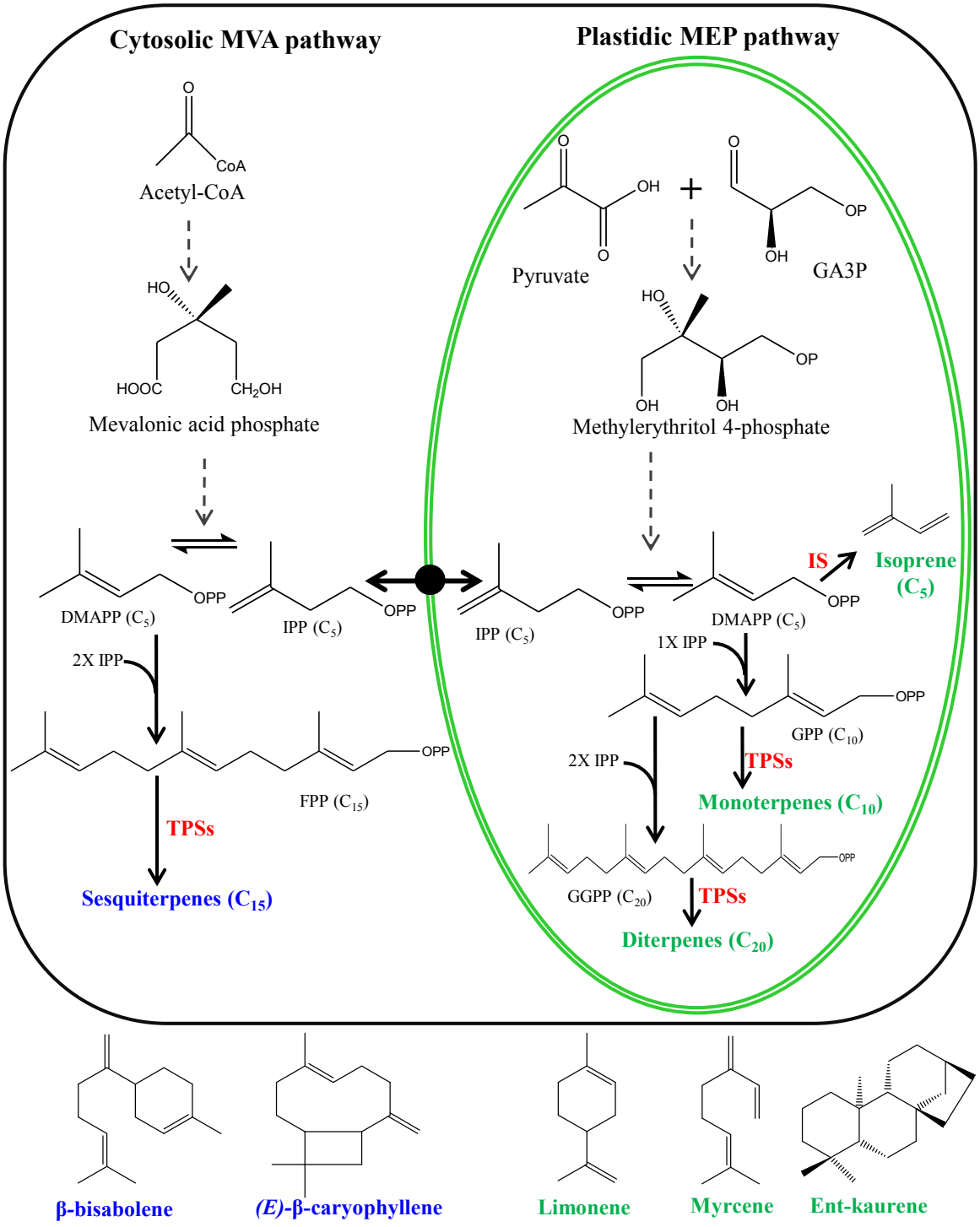

Fig. 1.2: A simplified biosynthetic pathway for the formation of terpenoids in plants. Both the cytosolic mevalonate **(MVA)** and the plastidial methylerythritol phosphate **(MEP)** pathways lead to the formation of the C_5 basic building blocks: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The MVA pathway is responsible for the production of sesquiterpenes (C_{15}) whereas the MEP pathway is responsible for the production of hemiterpenes (C_5), monoterpenes (C_{10}) and diterpenes (C_{20}). Representative structures of the sesquiterpenes, monoterpenes, and diterpenes are shown at the bottom.

1.4 The aim of this thesis

The present thesis addresses the following three major questions:

1. *Does genetically enhancing sesquiterpene production in Arabidopsis improves the plant's direct defenses against herbivores? (Research Chapter I)*

Expression of maize sesquiterpene synthase genes in Arabidopsis has been employed to demonstrate the roles of sesquiterpene volatiles in attracting parasitoids and predators of herbivores. However, information on the direct effect of this sesquiterpene manipulation on herbivore performance and its immediate effect on the natural defence phenotype of the transgenic plants is scarse. The objective of *Research Chapter I* was to investigate the performance of *Spodoptera littoralis* larvae on sesquiterpene overproducing transgenic Arabidopsis plants. When negative performance was observed, we aimed to clarify whether the sesquiterpenes directly affected larval performance or indirectly elicited the existing natural defenses of the transgenic plants simulating the within-plant defense signaling shown by other induced volatiles.

2. *Do volatile sesquiterpenes protect plants from ozone-induced injury like isoprene? (Research Chapter II)*

Isoprene and few monoterpenes have been demonstrated to protect plants from stresses caused by high temperature and ozone exposure. Sesquiterpenes share the same biosynthetic route and chemical properties with isoprene and monoterpenes, yet their role in thermotolerance and protection from ozone damage is less investigated. Although few sesquiterpene volatiles have been proposed to be efficient cleaners of ozone due to their high ozone reactivity, this interaction has not been studied from a phytocentric perspective. Therefore, in *Research Chapter II* we aimed to investigate whether two selected volatile sesquiterpenes, *(E)*-β-caryophyllene and *(E)*-β-farnesene, protect plants from ozone-induced damage when Arabidopsis plants are genetically engineered with the respective biosynthetic genes.

3. *Does restoring sesquiterpene production in a non-producing maize line protect the plant from multiple enemies? (Research Chapter III)*

Terpene manipulation in transgenic plants has been proposed to improve the resistance of crop plants against targeted herbivore enemies. Transgenic Arabidopsis and

maize plants overproducing sesquiterpene volatiles have been demonstrated to improve their indirect defenses by attracting natural enemies of the herbivores. However, less is known whether this enhancement of sesquiterpene production protect the plant from multiple pests. Previously, it has been shown that genetically restored maize line for the production of (*E*)-β-caryophyllene to sustain reduced herbivore damage due to the orchestration of indirect defense. In *Research Chapter III* we investigated the response of this same transgenic maize line to infection by the hemibiotrophic fungus *Colletotrichum graminicola*.

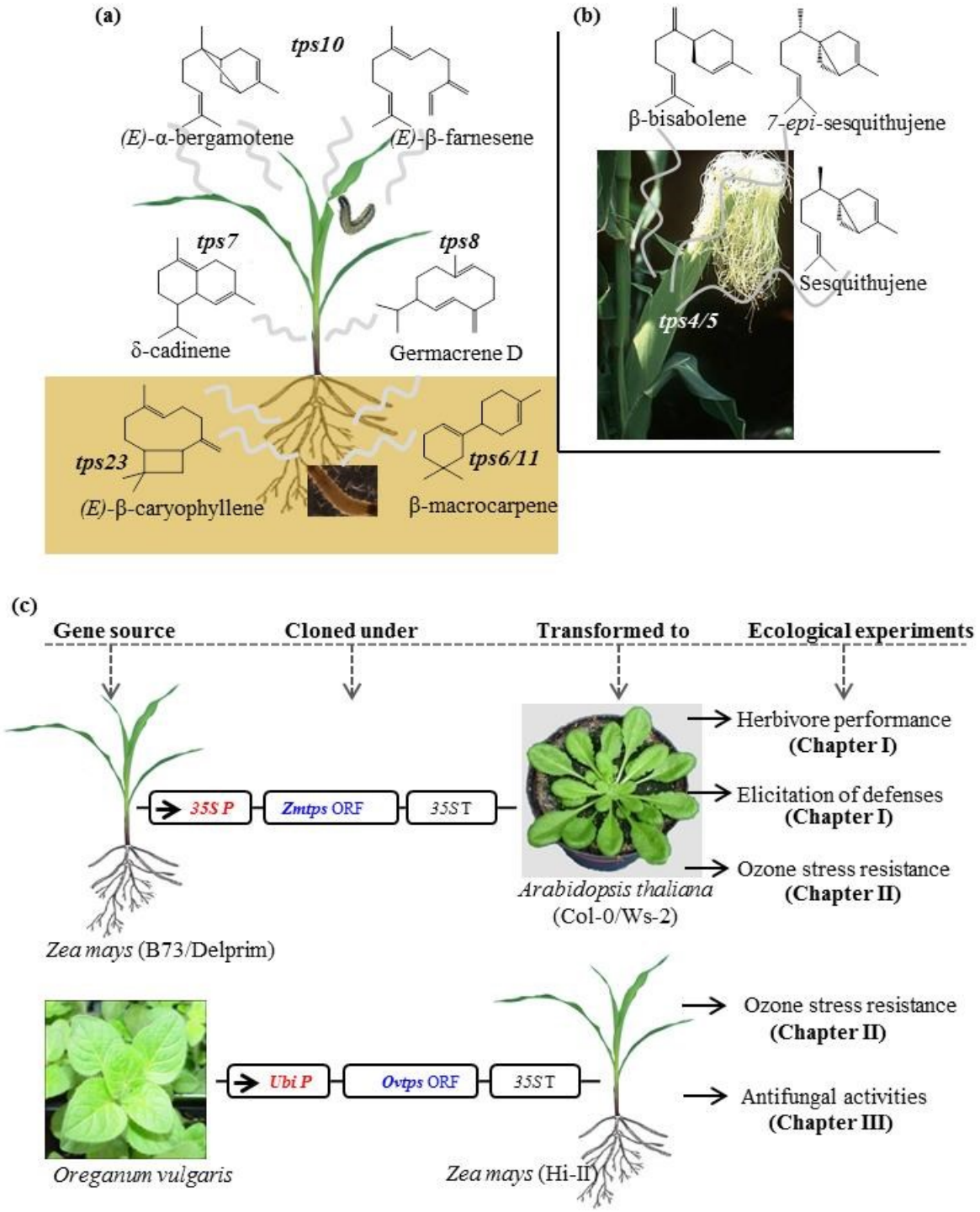

Fig. 1.3: Graphical representation of the study conducted in this thesis. Experiments were carried out to investigate the ecological roles of volatile sesquiterpenes. The tested sesquiterpene metabolites were originally identified in maize (*Zea mays*) where their biosynthesis was investigated. Some are constitutively emitted from young seedlings or emitted after herbivore attack above ground or below ground **(a)**. Others are emitted at later developmental stages especially from the husks **(b)**. Individual sesquiterpene synthase genes isolated from maize or oregano was used to transform Arabidopsis or non-emitting maize lines **(c)**. These transgenic plants were then used to test the roles of the volatiles in plant-herbivore interaction, ozone stress resistance and antifungal activities.

2. Research Chapter I

Volatile sesquiterpenes prime and elicit direct defenses in genetically engineered Arabidopsis plants*

Abstract

The role of plant volatiles in defense against herbivores has been well studied, but much less information is available about their role in intra-plant signaling that activates defense responses. The nature of the volatiles involved, the defenses activated and the molecular mechanism of volatile-mediated signaling are all poorly known. In this study we used transgenic *Arabidopsis thaliana* overexpressing maize sesquiterpene synthase genes to test the potential of the sesquiterpenes to defend plants against larvae of the generalist lepidoptera, *Spodoptera littoralis*, and to examine their role in intra-plant defense signaling. We assessed larval performance and behavior on transgenic vs. wild-type lines and analyzed the plants before and after herbivory for levels of chemical defenses and defense signaling molecules. *S. littoralis* larvae performed poorly on sesquiterpene-emitting lines. However, this poor performance was not due to a direct olfactory deterrent effect of the sesquiterpenes themselves. Instead, the sesquiterpenes were found to elicit the further induction of defenses already present in Arabidopsis including glucosinolates and proteinase inhibitors. Additionally the transgenic plants showed an increase in the baseline concentration of jasmonates and accumulated larger transcripts of selected defense genes following herbivory. These results suggest that sesquiterpenes which are often emitted in response to herbivore damage can function as internal signals to prime the induction of anti-herbivore defenses.

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2.1 Introduction

The vegetative organs of plants emit a complex mixture of volatile organic compounds that usually increase in quantity and complexity after herbivore attack. From the first reports of herbivore-induced volatiles, researchers have focused on their roles in attracting herbivore enemies. Over 50 different plant species are known to emit volatiles after herbivory that attract predators and parasitoids of herbivores (Turlings & Wackers, 2004; Mumm & Dicke, 2010). Volatile compounds are also known to directly repel herbivores from feeding and oviposition although this topic has received comparatively little attention (Unsicker et al., 2009). Mixtures of terpenes, the alkaloid nicotine, and green leaf volatiles (C_6 aldehydes, alcohols and esters derived from lipoxygenase cleavage of fatty acids) deterred the settling of aphids, whiteflies and thrips (Bernasconi et al., 1998; Beale et al., 2006; Delphia et al., 2007; Bleeker et al., 2009). In addition, certain lepidopteran species are repelled from ovipositing by blends of terpenes or green leaf volatiles (De Moraes et al., 2001; Kessler & Baldwin, 2001; Wang et al, 2008).

Apart from their role in direct and indirect defenses, plant volatiles have also been suggested to function as within-plant signals. Compounds synthesized and released after herbivore damage can act as cue for the presence of herbivores triggering the provision of defenses in the region of attack. For example, volatile blends released from damaged leaves of several species can cause adjacent leaves to increase their direct defenses to herbivores or increase the secretion of extra-floral nectar that attracts herbivore enemies (Karban et al., 2003, 2006; Frost et al., 2007; Heil & Bueno, 2007). In some instances, volatiles do not act to increase defenses immediately, but instead prime the organ to respond later when herbivores actually initiate their attack. These responses are greater in comparison to those in organs not exposed to volatiles (Frost et al., 2007; Rodriguez-Saona et al., 2009). Herbivore-induced volatile signaling among organs may be particularly significant in larger woody plants where vascular connections between organs are limited (Orians, 2005; Frost et al., 2007; Rodriguez-Saona et al., 2009), but could also be important over smaller distances in analogy to the way the volatile hormone ethylene acts. Herbivore-induced volatiles are generally lipophilic compounds and so could also move rapidly within the plant via membranes without being externally emitted.

Volatiles that signal within plants may also be detected by neighboring plants, and there is a long history of research involving volatile communication among plants.

Herbivore-induced volatiles have sometimes been reported to trigger defenses in neighbors or prime them to enhance their defenses after herbivory (Engelberth et al., 2004; Choh & Takabayashi, 2006; Kessler et al., 2006; Heil & Bueno, 2007; Ton et al., 2007; Frost et al., 2008a). However, doubts have been raised on the ecological relevance of such communication since volatile-induced defenses might improve the resistance of volatile "receivers" without obvious benefits for the "emitters", unless they are close relatives (Heil & Karban, 2010; Karban et al., 2011). Moreover, volatile-mediated plant-plant communication seems only effective over very short distances (Karban et al., 2003).

To learn more about the roles of plant volatiles, additional research is needed with a variety of plant species and different compound classes. In particular, more work should be carried out with terpenes, the most abundant group of plant volatiles (Unsicker et al., 2009; Mumm and Dicke, 2010). Most previous research has focused on green leaf volatiles, especially in studies on plant-plant communication (Engelberth et al., 2004; Farag et al., 2005; Ruther & Fürstenau, 2005; Kost & Heil 2006; Engelberth et al., 2007). Maize produces a large diversity of sesquiterpene volatiles (Köllner et al., 2004a&b), C_{15} terpenes that are products of a series of terpene synthases, including TPS1 (Schnee et al., 2002), TPS 4&5 (Köllner et al., 2004b), TPS 6&11 (Köllner et al, 2008b), TPS8 (Fontana, PhD Dissertation), TPS10 (Schnee et al., 2006) and TPS23 (Köllner et al., 2008a). These sesquiterpenes have been demonstrated to have a role in the attraction of herbivore enemies (Rasmann et al., 2005; Schnee et al., 2006; Köllner et al., 2008a; Fontana et al., 2011) and as precursors of anti-fungal compounds (Huffaker et al., 2011), but their possible functions in direct defense against herbivores and intra-plant communication have not been investigated. In addition, most of the work has focused on herbivore-induced sesquiterpene volatiles (Schnee et al., 2002; 2006; Köllner et al., 2008a&b), rather than those emitted constitutively (Köllner et al., 2004a&b).

There are a number of obstacles to testing the biological role of maize sesquiterpene volatiles. Most of the compounds produced are not commercially available and are difficult to synthesize in stereochemically pure forms. Moreover, since these substances are usually produced as complex blends that are the products of individual terpene synthases, they should ideally be tested as a blend away from the background of other maize volatiles. Since transgenic maize lines lacking most of their volatiles would be complex to engineer, we chose instead to introduce maize sesquiterpene synthases into a plant that does not normally release terpene volatiles. *Arabidopsis thaliana* does not

emit detectable levels of sesquiterpene volatiles at the rosette stage (Chen et al., 2003; Abel et al., 2009), but is able to produce sesquiterpenes readily when transformed with heterologous terpene synthases from maize (Schnee et al., 2006; Fontana et al., 2011) or other species (Aharoni et al., 2003; Kappers et al., 2005; Shiojiri et al., 2006). Moreover, transformation of Arabidopsis with terpene synthases results in plants that release the same compounds in the same proportions as found after expression *in vitro*.

In this chapter we investigated the roles of sesquiterpene volatiles in Arabidopsis transformed with two maize sesquiterpene synthases, *Zm*TPS4 and *Zm*TPS5, whose sesquiterpene products are constitutively emitted from the husks (Köllner et al., 2004a&b). We first determined the performance and behavior of larvae of the generalist herbivore *Spodoptera littoralis* on these transgenic Arabidopsis plants. After ascertaining that the larvae grew poorly on transformed plants and suffered increased mortality but were not deterred by the sesquiterpenes themselves, we looked for changes in other anti-herbivore defense compounds, including glucosinolate and proteinase inhibitors. These substances were elevated in plants transformed with the sesquiterpene synthases, but only after *S. littoralis* feeding. This suggests that the sesquiterpenes did not directly inflict the damage on the larvae but instead elicited the existing defense chemicals and proteins of Arabidopsis. To explore the mechanism of such defense elicitation, we investigated changes in phytohormone levels and gene expressions that are usually assessed in volatile primed plants.

2.2 Results

2.2.1 Metabolic engineering of sesquiterpenes in Arabidopsis

To study the defensive potential of the volatile sesquiterpenes of maize we transformed two sesquiterpene synthase genes under the control of a constitutive promoter into a plant with no constitutive foliar sesquiterpene emission. The genes, *tps4 and tps5* (Köllner et al., 2004b), were introduced into Arabidopsis (Col-0 accession) under the control of the *35S* Cauliflower mosaic virus (CaMV) promoter (Fig. 2.1a). The encoded terpene synthases are responsible for the production of the characteristic sesquiterpene blends found in the husks of maize plants. Both of these enzymes produce nearly the same products from the substrate farnesyl diphosphate, but in very different proportions (Fig. 2.1b). Among the major compounds formed are three pairs of bicyclic

sesquiterpenes with one member of each pair dominant in the blend of each terpene synthase. Thus transgenic lines with the *35S::tps4* constructs emitted large amounts of 7-*epi*-sesquithujene, *(E)*-α-bergamotene, and sesquisabinene A. Whereas, transgenic lines with the *35S::tps5* constructs emitted large amounts of the corresponding stereoisomers, sesquithujene, *(Z)*-α-bergamotene, and sesquisabinene B. In addition, both genotypes produced the monocyclic β-bisabolene and the acyclic *(E)*-β-farnesene in similar proportions.

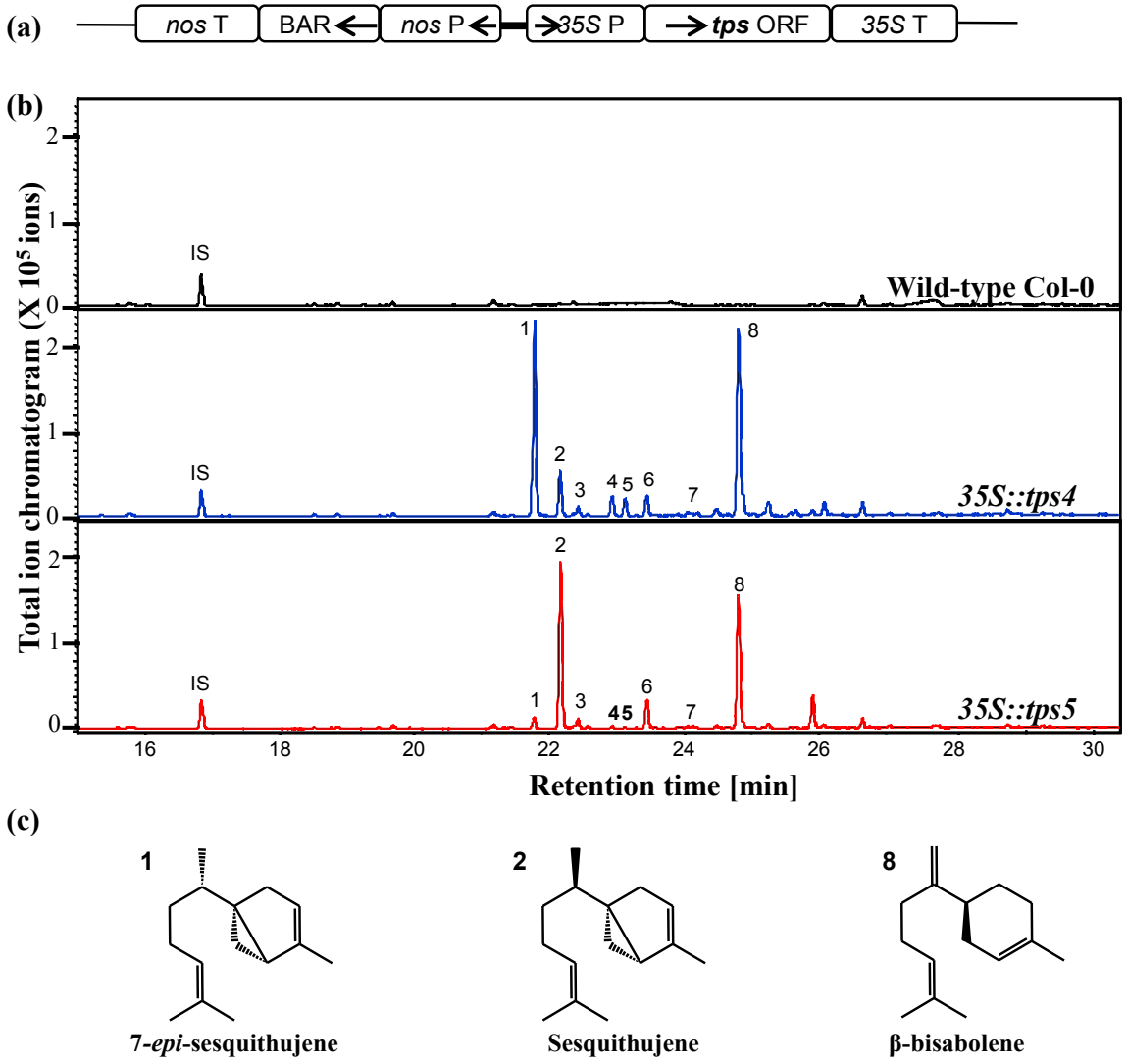

Fig. 2.1: Engineering Arabidopsis plants that express maize sesquiterpene synthase genes. **(a)** *35S::tps* construct scheme used to transform Arabidopsis. *nos* T-nopaline synthase terminator, *nos* P-nopaline synthase promoter, BAR-Basta resistance gene, *tps* ORF-terpene synthase open reading frame. **(b)** Headspace volatile collections from six week-old rosette leaves of Arabidopsis analyzed with GC-MS. The sesquiterpene products identified in the transgenic plants are as follows: 1. 7-*epi*-sesquithujene; 2. Sesquithujene; 3. *(Z)*-α-bergamotene; 4. *(E)*-α-bergamotene; 5. Sesquisabinene A; 6. Sesquisabinene B; 7. *(E)*-β-farnesene; and 8. β-bisabolene. Nonyl acetate was used as internal standard (IS). **(c)** Structures of the three major sesquiterpenes of the blend.

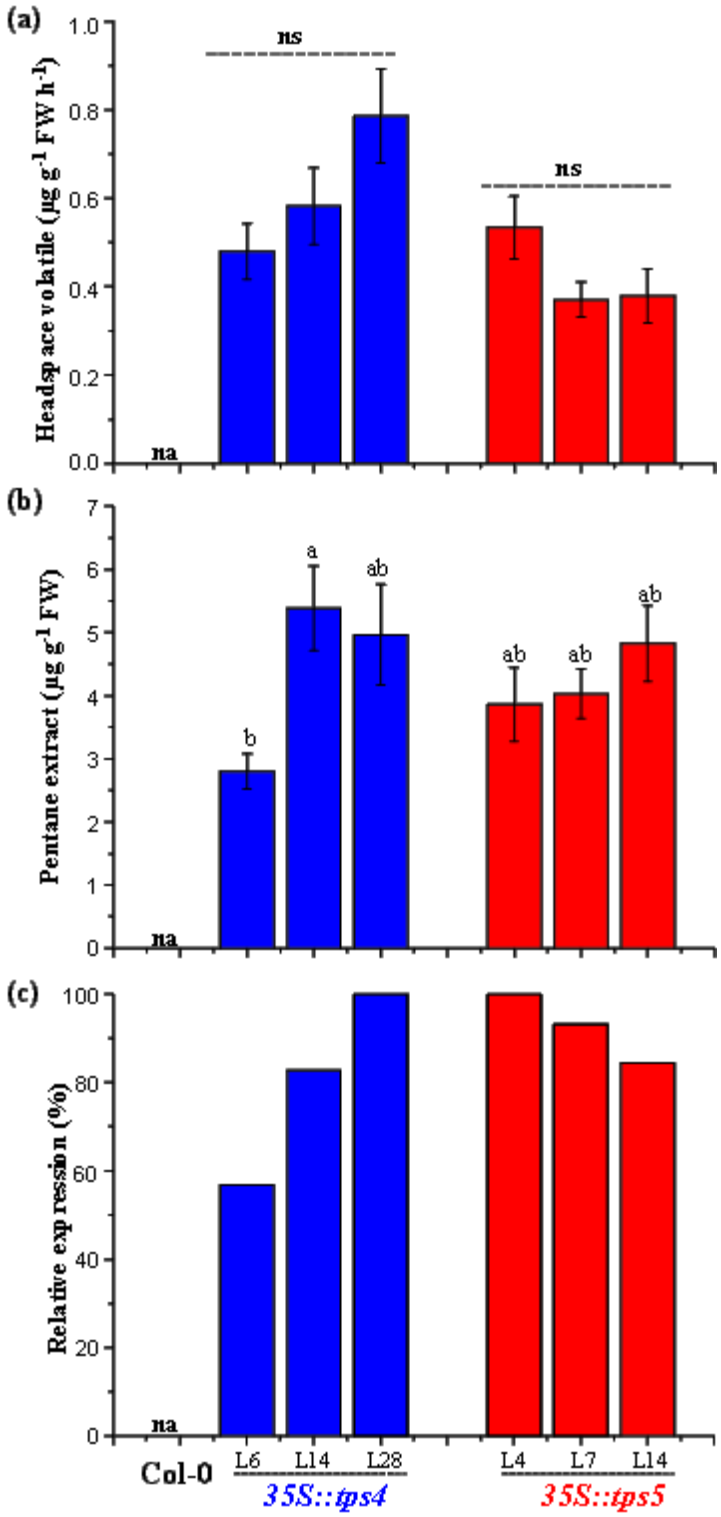

Fig. 2.2: Characterization of transgenic Arabidopsis lines for sesquiterpene production and transgene expression. **(a)** Headspace volatile collection and quantification of the total amount of the TPS4- and TPS5-type sesquiterpene products. The FID response was related to the nonyl acetate internal standard. The bars depict the mean ± SE (n = 10-17 replicates) **(b)** Internal sesquiterpene concentration as extracted by pentane and quantified like (a). Bars show mean ± SE (n = 15-17 replicates). **(c)** Transcript levels of *tps4* and *tps5* genes relative to the highest expressing lines, L28 and L4. Different letters on each bar represent significant differences after ANOVA followed by the Tukey HSD post hoc test at $\alpha = 0.05$.

However, wild-type control plants emitted none of these sesquiterpenes at the rosette stage (Fig. 2.1b). The total emission rate of the sesquiterpenes from the transgenic lines was between 0.4 and 0.8 $\mu g\ h^{-1}\ g^{-1}$ fresh weight (Fig. 2.2a). The concentration of these substances in leaf tissue, as determined by pentane extraction, was found to range between 4 and 8 $\mu g\ g^{-1}$ fresh weight (Fig. 2.2b). A correlation between the expression of the transgenes and the rate of sesquiterpene emission was observed (Fig. 2.2c). Three independently transformed lines of each construct were chosen for further experimental analysis.

2.2.2 Spodoptera larvae performed poorly on the transgenic lines

To evaluate the performance of *S. littoralis* larvae on the sesquiterpene-producing transgenic lines, we conducted a long-term feeding bioassay. The growth rate of the larvae was significantly reduced on these lines compared to the larvae feeding on wild-type plants (Repeated Measures ANOVA: $F_{6,826} = 12.58$, $p < 0.001$; Fig. 2.3a). This poor growth of the larvae was highly correlated with the sesquiterpene emission rates of the plants (2.3b). In addition to a decrease in growth, larvae feeding on the transgenic lines also suffered an increase in mortality (Survivor analysis with Cox's proportional hazards model with censoring, χ^2 (6, N = 140) = 15.46, $p = 0.017$; Fig. 2.3c). Within the first five days of feeding, 10 to 30 percent of the larvae feeding on the transgenic lines died; while 100 percent of the wild-type feeders remained alive. On the last day of censusing, up to 55 percent of the larvae fed on the transgenic lines died, while more than 80 percent of the larvae feeding on the wild-type were alive. Additional developmental parameters of the insect on these plants were also analyzed (Supplementary section, Table S9.1.2).

When given a choice to feed, *S. littoralis* larvae consumed on average 50 percent less leaf area on the transgenic lines compared to the wild-type during a 24 h feeding period (Fig. 2.4a). The amount of leaf area consumed was negatively correlated with sesquiterpene emission rates of the plants (Fig. 2.4b). To examine whether this negative performance of the larvae was due to an olfactory or gustatory effect, we performed a short-term orientation assay in which the larvae had to make a choice before getting into contact with the plant. As a result, the larvae did not make any olfactory preference between volatile cues from the transgenic or wild-type plants (Fig. 2.4c). On average, 38 percent of the larvae orientated towards wild-type plants and 35 percent towards the transgenic lines. Since the sesquiterpenes themselves did not deter feeding, we hypothesized that the

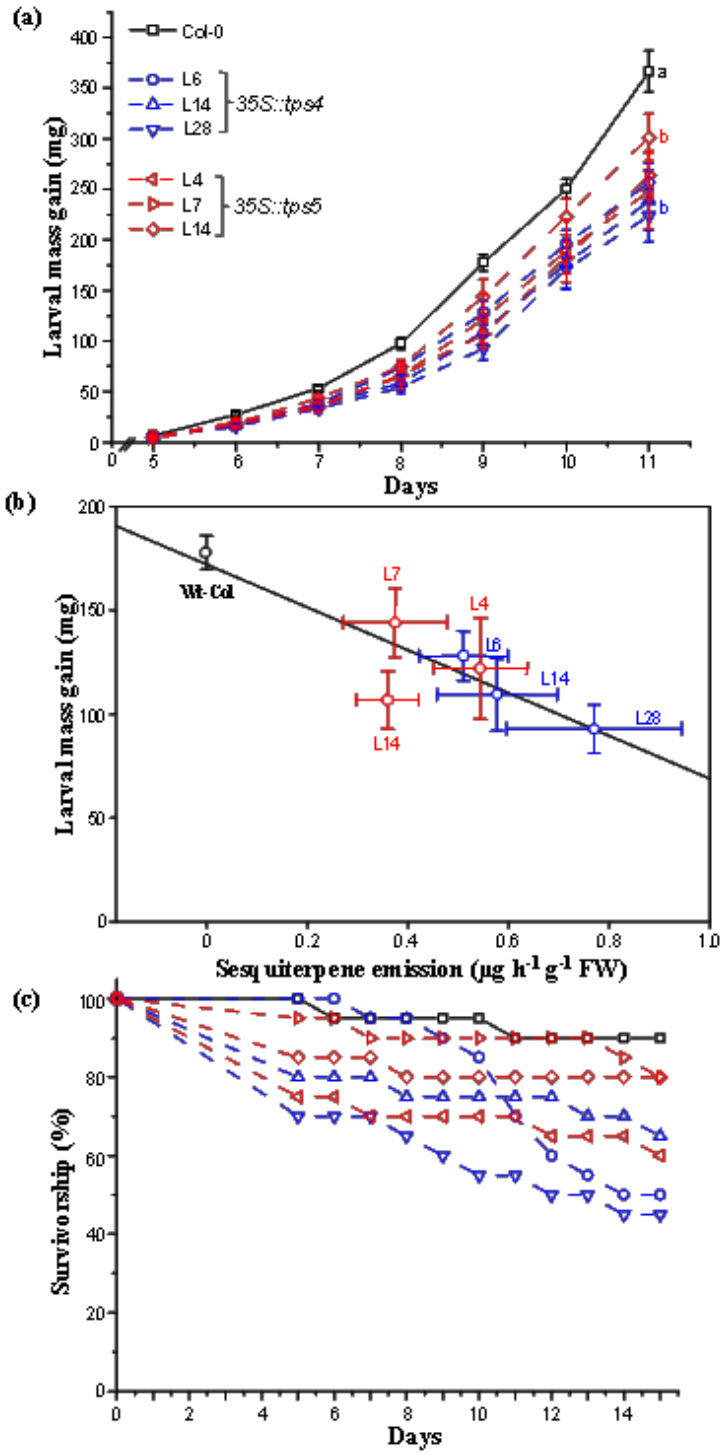

Fig. 2.3: *S. littoralis* larvae performance on sesquiterpene overproducing transgenic lines. Neonates of larvae which were hatched on artificial diet were allowed to feed on the plants. Five days after the onset of the experiment, larval mass and number of survivors were recorded daily until pupation. **(a)** Shows mean larval mass gain ± SE (n = 20 replicates). The mass gain of the larva over time was analyzed with Repeated Measures ANOVA model and different letters on the graph represent significant differences of the growth slopes on the different plant genotypes. **(b)** Relationship between the mean sesquiterpene emission rate of the different plant genotypes and *S. littoralis* larval mass gain at day 9. **(c)** Percent survivors (n = 20) over time. The mortality frequency of the larvae was analyzed using a Two-way contingency table analysis and significant differences are reported in the text.

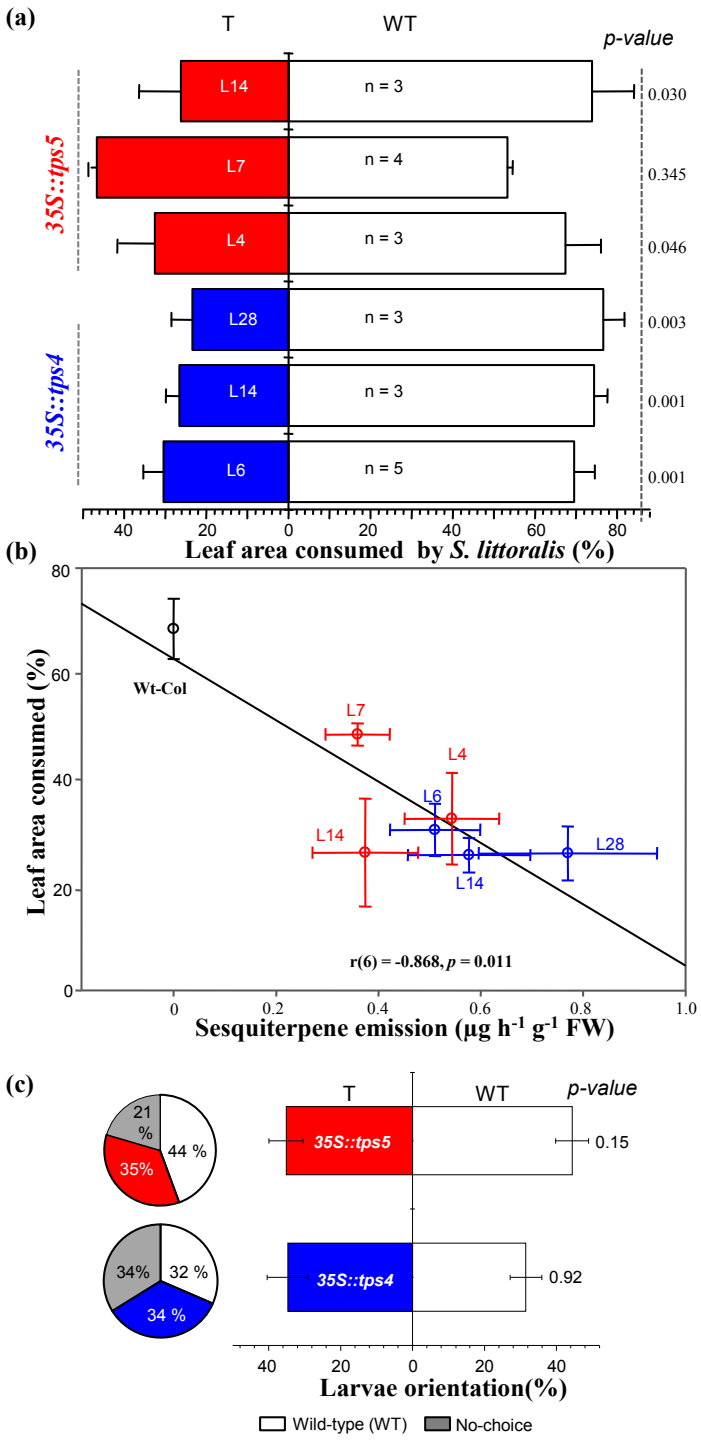

Fig. 2.4: Feeding preference and orientation of *S. littoralis* larvae on wild-type and transgenic Arabidopsis lines. **(a)** To assess the feeding preferences, third instar larvae were released in groups of ten to twenty in the middle of a choice arena and allowed to feed on alternately arranged wild-type (WT) and transgenic (T) plants for about 18 h. Leaf area consumed by the larvae on each line was estimated and percent leaf area consumed was calculated and used as an index of larval feeding preference. Percentage leaf area consumed ± SE of three to five independent experiments are shown. **(b)** Relationship between the leaf area consumed on each genotype and the amount of emitted sesquiterpenes. **(c)** To test the orientation of the larvae solely with olfactory cues from WT and T plants, a modified choice arena was used. Percent larvae attracted to either a WT or T volatile cues ± SE of four independent experiments are shown. The p-values are based on independent samples t-test.

transgenic plants might be in a primed state or elicited other defense phenotypes that could potentially cause the poor performance of the larvae. Therefore, we characterized the transgenic lines for alterations commonly associated with the primed state of plants, including defense metabolite accumulation, gene expression, and changes in phytohormone profiles.

2.2.3 Sesquiterpene expression in Arabidopsis enhanced glucosinolate concentrations

Glucosinolates are amino-acid derived defense compounds which are commonly found in the Brassicaceae family including the model plant Arabidopsis. In order to understand the cause for the poor performance of *S. littoralis* larvae on the terpene overproducing transgenic lines, we targeted to analyze this major defense metabolite in Arabidopsis. We identified four indole and six aliphatic glucosinoltae types from rosette leaves. Herbivory by *S. littoralis* larvae induced the accumulation of most of the glucosinolate types in all the plant genotypes but the degree of induction was larger in the transgenic plants compared to the wild-type (Fig. 2.5). While the total glucosinolate concentration increased by 9 percent in wild-type plants, this level was 14 to 43 percent higher in the transgenic lines following a 24 h herbivory period (Fig. 2.5a). When the foraging time was extended to 48 h, the increase was 32 percent for the wild-type and 48 to 85 percent for the transgenic lines. Interestingly, the baseline concentration of most of the indole glucosinolates (Fig. 2.5e-g) and one of the short-chain aliphatic glucosinolates (Fig. 2.5i) seem to be larger in the transgenic lines than the wild-type although statistical difference was not observed for the indoles. The three indole glucosinolate types that were found in significantly larger concentration in the transgenic lines at both time points were glucobrassicin (I3M) (Fig. 2.5d), 4-hydroxyglucobrassicin (4OHI3M) (Fig. 2.5e), and neoglucobrassicin (1MOI3M) (Fig. 2.5g). However, the fourth indole glucosinolate type, 4-methoxyglucobrassicin (4MOI3M) did not show any induction but was constitutively present in larger amount in the transgenic lines (Fig. 2.5f). From the six aliphatic glucosinolates identified, only the two short-chain aliphatic glucosinolates, glucoraphanin (4MSOB) (Fig. 2.5h) and glucoallysin (5MSOP) (Fig. 2.5i) were differentially accumulated in both wild-type and transgenic lines. While 4MSOB was significantly induced by *S. littoralis* feeding in the transgenic lines at both feeding time

points, 5MSOP seemed less responsive to herbivory but accumulated in significantly larger concentration in the transgenic lines before herbivore challenge.

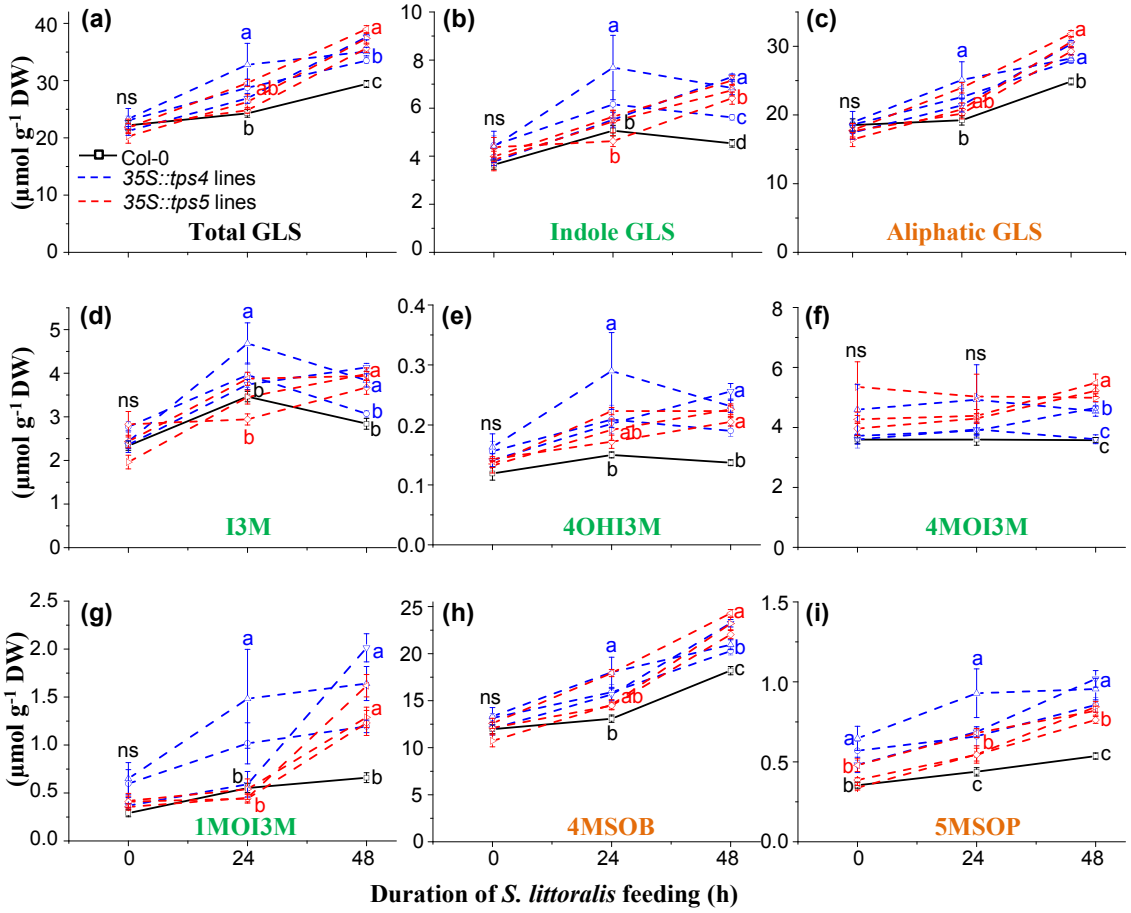

Fig. 2.5: Comparison of glucosinolates (GLS) concentration in wild-type and sesquiterpen overproducing transgenic Arabidopsis. Six-week old rosette leaves were collected from undamaged and Herbivore-damaged (24 and 48 h *S. littoralis* feeding) plants and flash-frozen in liquid nitrogen. Glucosinolates were extracted with 80 percent methanol containing p-hydroxybenzyl glucosinolate as internal standard and analyzed with HPLC after desulfating the glucosinolates with sulfatase. **(a)** Total glucosinolates, **(b)** sum of indole glucosinolates, **(c)** sum of aliphatic glucosinolates, **(d-g)** indole glucosinolates, and **(h-i)** aliphatic glucosinolates. The graphs represent mean ± SE (n = 6-10 replicates) and different letters on the graphs represent significant differences after ANOVA followed by Tukey HSD post hoc test at $\alpha = 0.05$. Whenever possible, lines of each genotype were combined and treated as one factor for ANOVA.

2.2.4 Sequiterpene expression in Arabidopsis enhanced defense gene expressions

In addition to glucosinolates, the expression of two key defense proteins in Arabidopsis, vegetative storage protein2 (*VSP2*) and Arabidopsis trypsin proteinase inhibitors (*ATTIs*) were also analyzed. Both proteins are known for their insect-

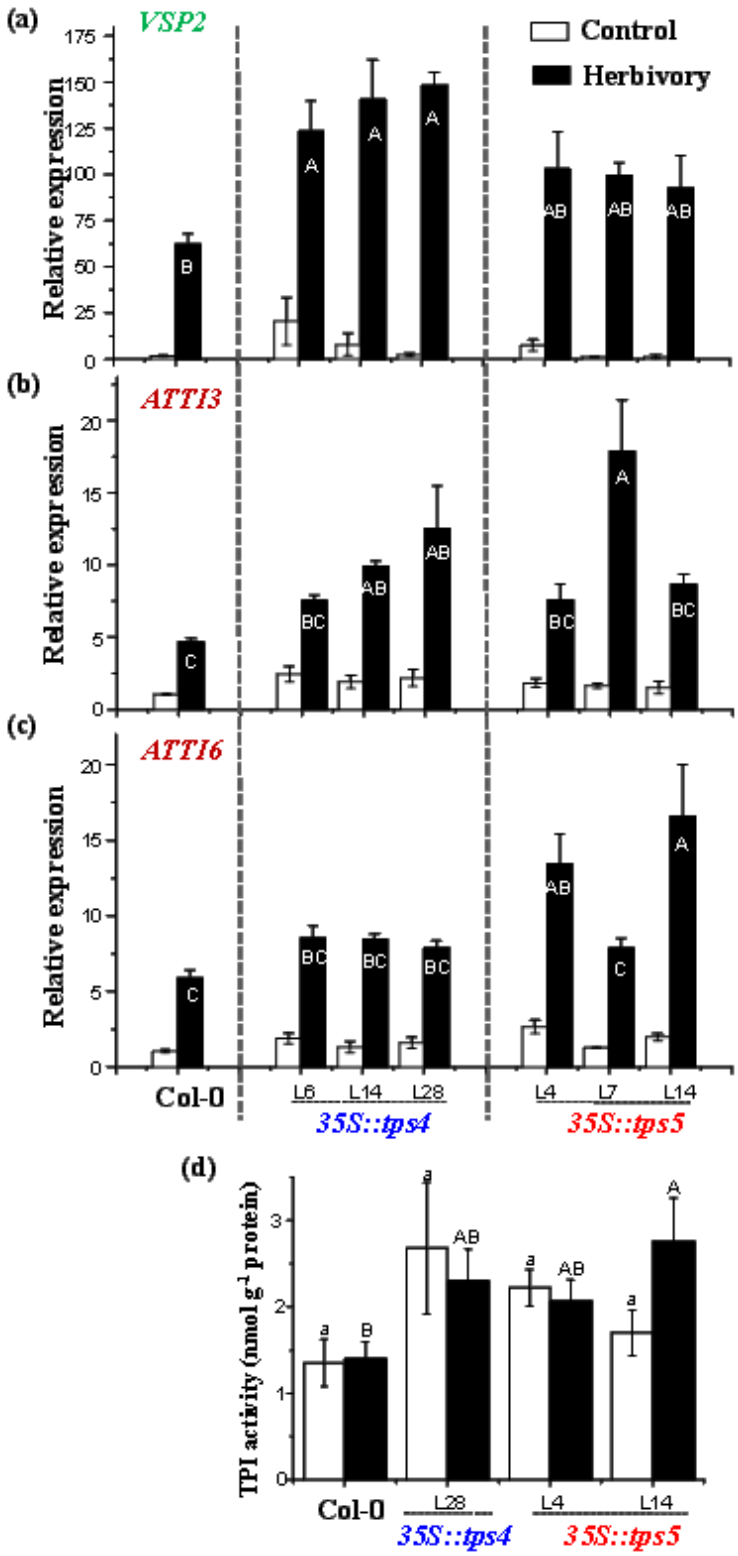

Fig. 2.6: Expression analysis of selected defense genes in Arabidopsis. The expression of vegetative storage protein2 (*VSP2)* **(a)** and Arabidopsis trypsin proteinase inhibitor (*ATTI)* **(b,c)** genes were analyzed after the plants were induced by *S. littoralis* feeding for a period of 24 h. qRT-PCR was performed on three biological replicates, normalized to *ACTIN* gene and calibrated with undamaged wild-type plants. Proteinase inhibitor activity was assayed on the crude protein extracts of the samples **(d)**. The graphs represent the mean ± SE values. Bars with different letters represent significant differences after ANOVA followed by Tukey HSD post hoc test at $\alpha = 0.05$.

inducibility and potent antiherbivore activity (Cipollini et al., 2004; Clauss & Mitchell-Olds, 2004; Liu et al., 2005). In undamaged plants, the transcript levels of *VSP2* and two trypsin proteinase inhibitor genes *ATTI3* and *ATTI6* were low and no significant difference was observed between wild-type and transgenic plants (Fig. 2.6a-c). However, feeding by *S. littoralis* larva for a period of 24 h increased the expression of these genes and the transcript accumulation of both genes was larger in the transgenic lines. The expression of *VSP2* in the *35S::tps4* lines was significantly larger compared to the wild-type (Fig. 2.6a). A similar trend was also observed in the *35S::tps5* lines though significant statistical difference was not observed. The expression of the two candidate proteinase inhibitors in Arabidopsis was also generally found significantly larger in most of the transgenic lines compared to the wild-type plants (Fig. 2.6b,c). We also determined the *in vitro* proteinase inhibitor activity of the crude protein extracts of the plants that were subjected to *S. littoralis* herbivory for a period of 24 h. Interestingly the transgenic lines were found to have larger preformed proteinase inhibitor activity than the wild-type plants (Fig. 2.6d).

2.2.5 Concentration of jasmonic acid and isoleucine conjugates are higher in the transgenic lines

Priming by volatiles usually enhances the accumulation of jasmonic acid (JA) which further controls the activation of genes encoding antiherbivore defense proteins and metabolites (Engelberth et al., 2004; Ton et al., 2007; Kishimoto et al., 2006; Frost et al., 2008a). In order to understand whether the observed increase in the glucosinolate concentration and defense gene expressions in the transgenic lines were associated with altered jasmonate level; we targeted to analyze JA which is commonly involved in signaling processes that are associated with herbivory. Interestingly, the baseline concentration of both JA-isoleucine conjugates, (-)-JA-L-Ile (Fig. 2.7b) and (+)-*7-iso*-JA-L-Ile (Fig. 2.7c) were found in significantly larger amounts in the transgenic lines compared to the wild-type. The baseline concentration of JA itself was found to be significantly larger in the lines overexpressing the TPS4-type sesquiterpenes (Fig. 2.7a). Although the baseline increase in JA and its isoleucine conjugates is a typical phenotype of the mutants (Fig. S9.1.2), the transgenic lines did not show any alteration in other types of phytohormones (Fig. S9.1.3). Feeding by *S. littoralis* larvae for a period of 24 h

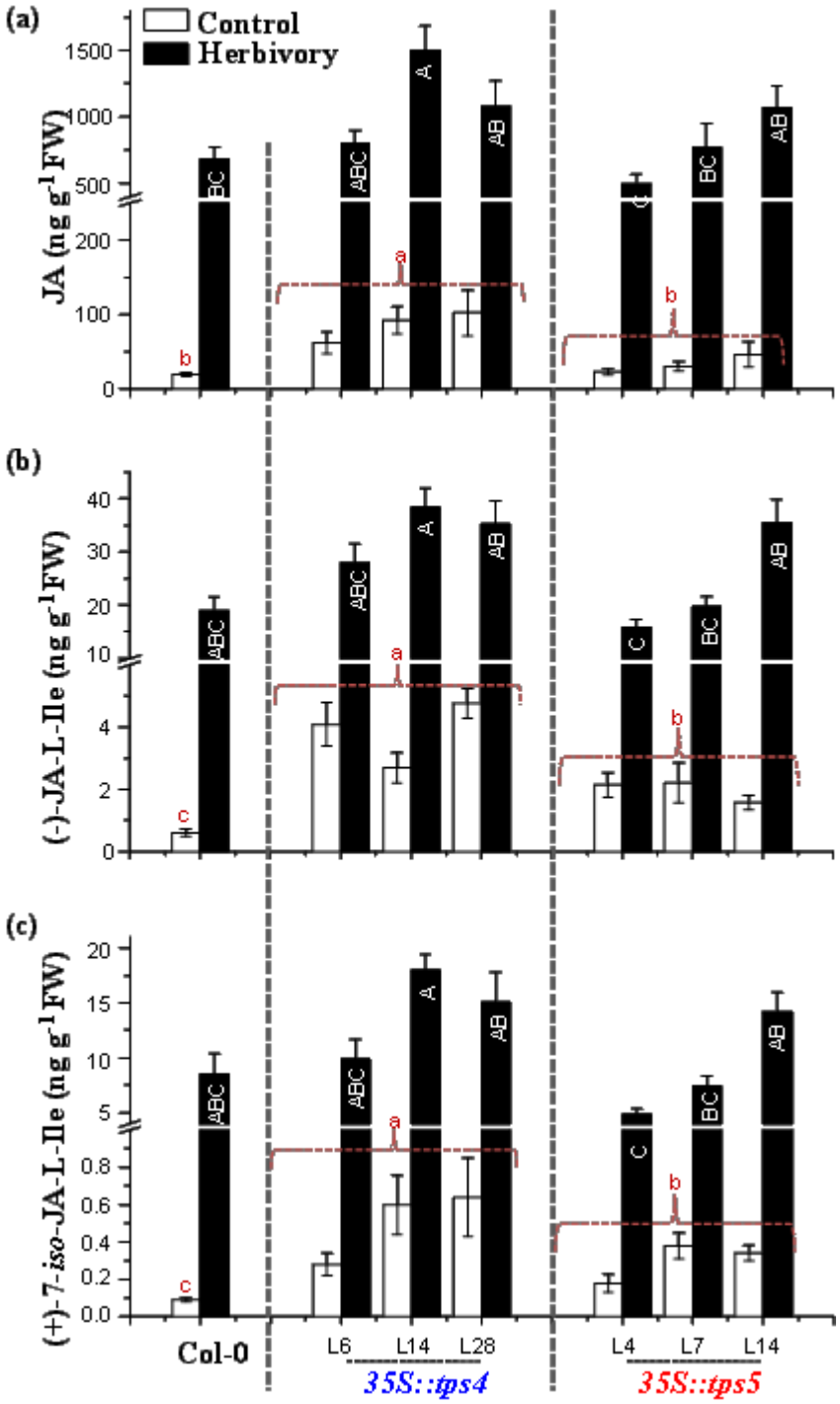

Fig. 2.7: Comparison of the baseline and herbivore-induced jasmonate concentrations in the transgenic and wild-type Arabidopsis plants. Third instar *S. littoralis* larvae were allowed to feed on six-week old Arabidopsis rosettes for 24 h. Herbivore-damaged rosette leaves were then harvested and flash-frozen in liquid nitrogen. The phytohormones were extracted with pure methanol containing JA and JA-Ile internal standards. The extracts were analyzed with a triple quad LC/MS/MS instrument. **(a)** JA, **(b)** (-)-JA-L-Ile, and **(c)** *(+)-7-iso*-JA-L-Ile. Bars represent mean ± SE (n = 10-16 replicates). Different letters on each bar represent significant differences after ANOVA followed by the Tukey HSD post hoc test at $\alpha = 0.05$.

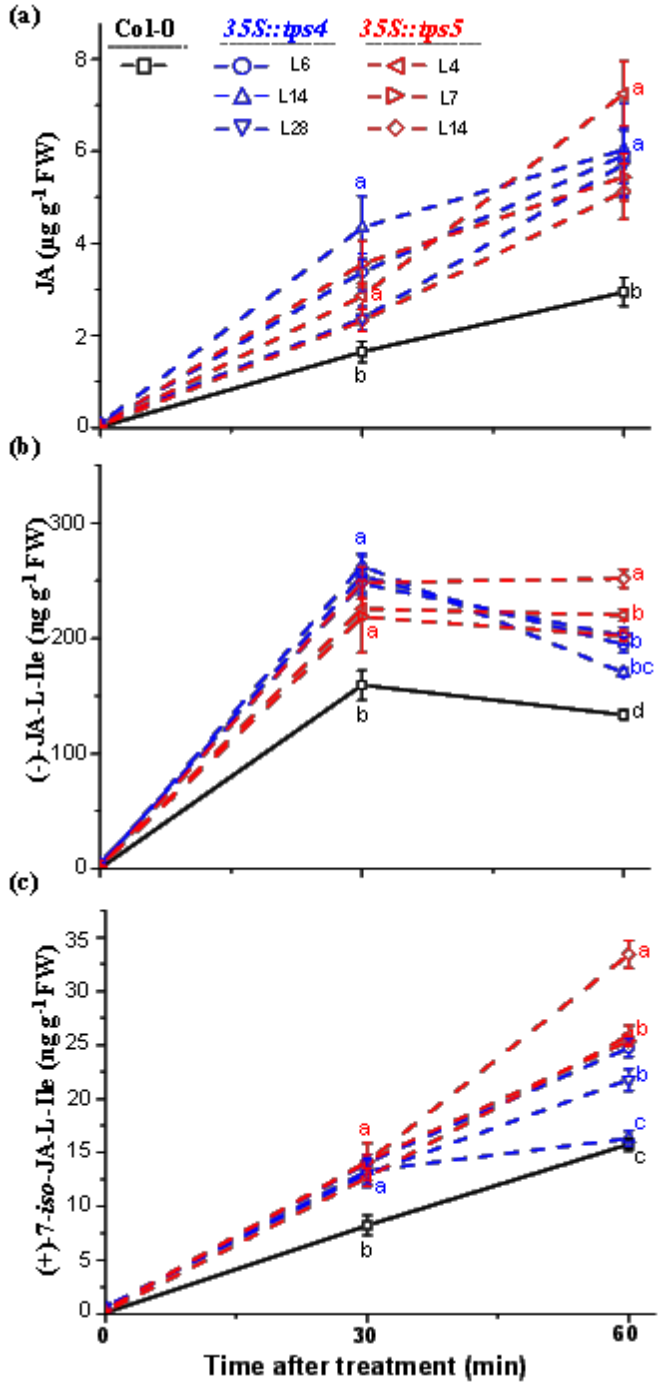

Fig. 2.8: Analysis of short-term transient jasmonate responses in transgenic and wild-type Arabidopsis after simulated herbivory. A pattern wheel was used to mechanically wound leaves along mid-veins. On the wounded sites 1:5 diluted *S. littoralis* oral secretion was applied. The phytohormones were extracted and analyzed at 30 and 60 minutes after elicitation and compared with the concentrations of undamaged control plants represented at the 0 min time point. **(a)** JA measurement, **(b)** (-)-JA-L-Ile measurement, and **(c)** (+)-7-iso-JA-L-Ile measurement. The graphs represent the mean value ± SE of three replicates. Different letters on the graphs represent significant differences after ANOVA following Tukey HSD post hoc test at $\alpha = 0.05$. Whenever possible, the ANOVA model was simplified by grouping lines in one genotype.

increased the levels of these hormones 20- to 24-fold in all plant genotypes with no statistical difference between the wild-type and the transgenic lines (Fig. 2.7). Since most priming phenomena affect the kinetics of the initial responses, we assessed the phytohormone concentrations at 0, 30, and 60 minutes after simulated herbivory by mechanically wounding rosette leaves and applying *S. littoralis* oral secretion. The levels of the jasmonates were dramatically increased at 30 and 60 minutes after elicitation and the induction was significantly larger in the transgenic lines compared to the wild-type plant (Fig. 2.8). This kinetics of the jasmonates suggests that the sesquiterpene-producing transgenic lines might be in a primed state.

2.2.6 LOX2 and AOS genes are highly expressed in the transgenic lines

To determine whether the increase in jasmonate concentration correlates with the expression of JA biosynthetic genes, the transcript abundance of lipoxygenase2 (*LOX2*) and allene oxide synthase (*AOS*) was determined. These enzymes catalyze the initial steps of the JA biosynthetic pathway and were shown to be affected by volatile priming in previous studies. In undamaged control plants, the transcript levels of *LOX2* (Fig. 2.9a) and *AOS* (Fig. 2.9b) did not differ significantly between the wild-type and transgenic lines. The transcript levels of both enzymes were increased 5- to 6-fold in the wild-type and 8- to 13-fold in the transgenic lines after 24 h continuous feeding by *S. littoralis* larvae. Generally, the expression of both genes were significantly larger in most of the *35S::tps4* lines compared to the wild-type; but these values showed increased trend in the *35S::tps5* lines with no statistical difference, except for line7 in *LOX2* expression (Fig. 2.9a,b). When the kinetics of transcript accumulation was assessed at 0, 30, and 60 min after simulated herbivory, the expression of *LOX2* peaked 30 min after elicitation in two of the sesquiterpene-producing lines while the wild-type plants showed only little response (Fig. 2.9c). Similarly, the expression of *AOS* was significantly higher in the transgenic lines at both 30 and 60 min time points after elicitation than the wild-type (Fig. 2.9d).

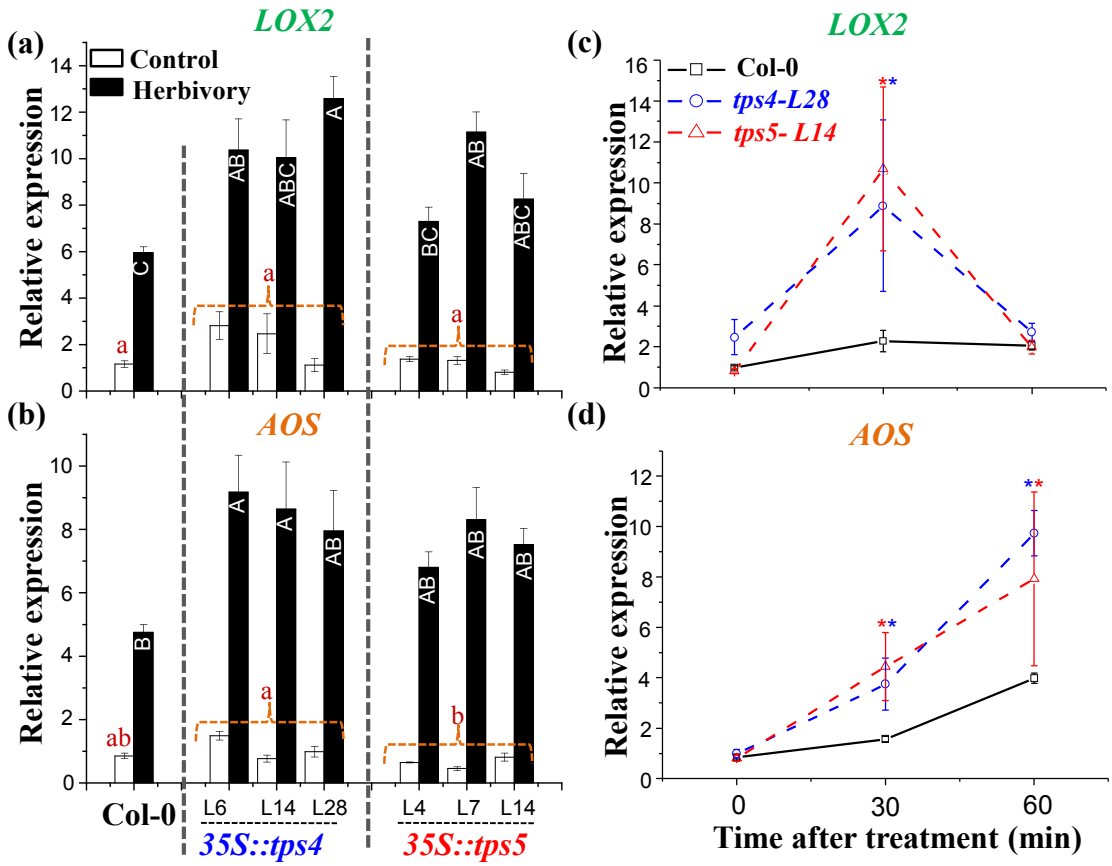

Fig. 2.9: Transcript levels of selected genes in the JA biosynthetic pathway. Relative transcript accumulation of **(a)** *LOX2* and **(b)** *AOS* were compared after 24 h *S. littoralis* feeding in wild-type and transgenic Arabidopsis lines. A short-term time course responses of *LOX2* **(c)** and *AOS* **(d)** were also analyzed after simulated herbivory with mechanical wounding and *S. littoralis* oral secretion application. The qRT-PCR experiments were conducted with three technical and three biological replicates, normalized to ACTIN gene and calibrated with undamaged wild-type plants. The graphs represent the mean ± SE of three biological replicates. Bars with different letters or asterisks represent significant differences after ANOVA followed by the Tukey HSD post hoc test at $\alpha = 0.05$.

2.3 Discussion

The ability of plants to perceive volatile signals and subsequently alert their defense status seems to be an adaptive mechanism. However, the majority of evidence on volatile signaling comes from experiments in which the signal is artificially directed to receiver plants in an airtight enclosure. In addition, in identifying the actual volatile component that primes a defense response, most experiments used only synthetic GLVs. Under attack by herbivores, plants usually emit not only GLVs but also a complex blend of other volatiles dominated by terpenoids. Examining transgenic plants that produce a particular volatile component will help to dissect whether volatiles prime and induce defenses when they are internally generated. In the current study we utilized Arabidopsis

plants that are transformed with sesquiterpene biosynthetic genes to study the potential of sesquiterpenes as endogenous signals to prime and induce defenses. We used insect behavioral and physiological responses and plant biochemical and molecular analysis to measure the defense status of the transgenic plants that produce the sesquiterpenes.

The initial observation that the performance of the larvae reared on the sesquiterpene-producing Arabidopsis highly impaired (Fig. 2.3) prompted us to investigate the plant's defense response in more detail. When given a choice, the larvae preferably fed on wild-type plants. However, when larval choice tests were based solely on volatile cues, there were no preferences (Fig. 2.4c). This suggests that the poor performance of the larvae is not due to a direct olfactory deterrence effect of the sesquiterpenes. This finding is supported by previous observations that most polyphagous caterpillars have poor olfactory threshold levels and do not have an innate olfactory response to foodplant selection (Dethier 1939; Carlsson, 1999). Thus, we suggest that the poor performance of the larvae might be caused by postingestive disorders. One can also argue that the ingested sesquiterpenes in the context of transgenic plant tissue may cause a direct toxic effect in the gut of the larvae. However, this is unlikely because these sesquiterpenes are not stored in larger quantities in the plant and accumulate only to concentrations below 5 $\mu g\ g^{-1}$ fresh weight. These concentrations of sesquiterpenes are unlikely to affect a generalist herbivore like *S. littoralis*, as has been shown in previous studies (Srivastava et al., 1990; Gonzalez-Coloma et al., 1995; Frelichowski and Juvik, 2001). Therefore, we argue that the transgenic plants might mobilize other endogenous defensive traits that could potentially cause the poor performance of the larvae.

Since glucosinolates are the major constitutive and induced defense metabolites in Arabidopsis (Textor & Gershenzon, 2009), we analyzed these compounds to check if the natural concentration and profile is altered in the transgenic lines. Our observation clearly indicated that the sesquiterpene-producing transgenic lines tended to accumulate a higher baseline concentration of most indole glucosinolates and one aliphatic glucosinolate (Fig. 2.5e-g,i) and feeding by *S. littoralis* larvae preferentially induced these levels in larger quantities in the transgenic lines compared to the wild-type (Fig. 2.5a). These results indeed suggest that the enhanced glucosinolate levels in the transgenic lines may partially caused the poor performance of the larvae. It is usually common to activate various direct and indirect defensive traits in plants that are exposed to volatiles. For instance, lima bean activates extrafloral nectar (EFN) secretion (Heil & Bueno, 2007), poplar increases

phenolic compounds and terpenoid volatiles (Baldwin & Schultz, 1983; Frost et al., 2007; 2008a), and wild tobacco increases nicotine levels (Kessler et al., 2006). Although we do not know the mechanism how these sesquiterpenes activate glucosinolate metabolism in Arabidopsis, the response may resemble those responses observed in other plants that are exposed to volatiles and activate various defensive traits in a species dependent manner.

In addition to the glucosinolates, Arabidopsis has other defensive traits which are commonly activated during herbivore attack. We chose to investigate the expression of two such direct defense proteins, the *Arabidopsis vegetative storage protein2* (*VSP2;* Liu et al., 2005) and the *Arabidopsis thaliana trypsin proteinase inhibitor* (*ATTI*; Clauss & Mitchell-Olds, 2004; Cipollini et al., 2004). The increased transcript accumulation of both defense proteins in the transgenic lines (Fig. 2.6) suggested that the poor performance of *S. littoralis* larvae may be attributed to both the enhanced glucosinolate metabolism and the activation of these potent defense proteins. Activation of proteinase inhibitor genes has been also described in maize plants that are exposed to terpene dominated conspecific herbivore-induced volatiles (Ton et al., 2007; Ali et al., 2013) and poplar fumigated with pure GLVs (Frost et al., 2008a). This enhanced direct defense phenotype has been signified by the poor performance of *S. littoralis* larvae reared on these plants (Frost et al., 2008a). Although Kessler et al (2006) did not observe any proteinase inhibitor activity in volatile exposed wild tobacco plants; Frost et al (2008a) found both increased transcript accumulation and enzyme activity in GLV exposed poplar plants. Arabidopsis itself can increase the expression of its *VSP2* gene following exogenous application of monoterpenes and GLVs (Kishimoto et al., 2006; Godard et al., 2008; Zhang et al., 2012).

Volatile exposed plants usually increase the accumulation of JA which subsequently regulates the activation of genes encoding antiherbivore defense proteins and metabolites (Ton et al., 2007; Kishimoto et al., 2006). The increase in the baseline concentration of JA and its isoleucine conjugates (Fig. 2.7) indicated that transgenic plants that express the volatile signals internally are able to activate JA like other plants that are exposed to the volatiles externally. This baseline increase in JA concentration might be responsible to the observed increases in the resting levels of some of the glucosinolate types identified (Fig. 2.5e-g,i) and the proteinase inhibitor activity (Fig. 2.6d). It is well known that JA regulates the downstream expression of proteinase inhibitor and VSP genes through a COI1 dependent activation of transcription factors (Koiwa et al., 1997; Takahashi et al., 2007) and recently it has been shown that

transcriptional responses of Arabidopsis in response to exposure to terpenes and GLVs is regulated through the COI1 signaling pathway (Zhang et al., 2012). The effect of the JA signaling pathway in regulating the constitutive level of glucosinolates, especially the indole glucosinolates, has been also best described in *coi1* mutant Arabidopsis lines (Mewis et al., 2005; 2006). Therefore, the observed increasing trend in the constitutive concentration of glucosinolates in the transgenic lines might be linked to the corresponding increase in the baseline concentration of jasmonates in these lines. However, we do not have any direct evidence that the observed increase in the herbivore-induced glucosinolate accumulation in the transgenic lines (Fig. 2.5) was also related to this baseline increase in the JA concentration. Although the herbivore-induced accumulation of glucosinolates depends on the type of herbivore feeding guild, Mewis et al (2006) reported that this induced glucosinolate accumulation is not regulated by the JA-COI1 signaling pathway. In a previous study, however, JA-dependent elicitor induced indole glucosinolates accumulation has been shown to be governed by the JA-COI1 signaling pathway (Brader et al., 2001).

One should not also exclude the possibility that the enhanced level of JA in the transgenic lines may directly or indirectly regulate the activation of other transcription factors and biosynthetic genes in glucosinolate metabolism in a COI1 independent manner. For instance, JA-dependent activation of *AtDof1.1* and *MYB29* transcription factors (Skirycz et al., 2006; Hirai et al., 2007) and *CYP79B2* and *CYP79B3* biosynthetic genes (Mikkelsen et al., 2003) have been shown to trigger the induction of indole and aliphatic glucosinolate types in Arabidopsis. Although Arabidopsis has been shown to activate some of its defensive traits after exposure to monoterpenes or GLVs (Kishimoto, et al., 2005; 2006; Godard et al., 2008), our work is the first to show glucosinolate enhancement in sesquiterpene overproducing transgenic Arabidopsis lines. However, it has been reported that Arabidopsis plants exposed to green leaf volatiles to increase their aliphatic glucosinolates faster than unexposed controls, though this report is solely based on personal communications without published data (Frost et al., 2008b).

The altered jasmonate profile bears a close resemblance to those observed for other plants that are either exposed to pure GLVs and monoterpenes (Engelberth et al., 2004; 2007; Frost et al., 2008a; Godard et al., 2008) or herbivore-induced VOC blends (Engelberth et al., 2004; Rodrigues-Saona et al., 2009). Although we could not infer the mechanism how JA is primed by the sesquiterpenes in Arabidopsis, JA priming by

volatiles in hybrid poplar has been suggested to be due to the activation of enzymes and precursor substrates upstream of the JA biosynthesis pathway (Frost et al., 2008a). A similar sort of self-priming with its naturally existing constitutive sesquiterpene has been recently described to enhance the baseline concentration of JA in a maize inbred line Mp708 (Shivaji et al., 2010; Smith et al., 2012). The authors suggest that this maize line might be in a primed state that renders resistance to the fall armyworm larvae (*Spodoptera frugiperda*).

Two phospholipase enzymes were highly induced by exogenous application of GLV, subsequently leading to the increased accumulation of linolenic acid and *LOX* expression. While Frost et al (2008a) did not find any activation of *AOS* in poplar, we observed induction of both *LOX2* and *AOS* genes in the transgenic Arabidopsis lines after herbivory and simulated herbivory (Fig. 2.9). This is in agreement with other findings, where changes in the transcription of genes involved in the JA biosynthesis were described after volatile exposure (Farag et al., 2005; Kishimoto et al., 2005; 2006; Engelberth et al., 2007; Godard et al., 2008). Although the physiological and molecular mechanism controlling VOC-induced priming and defense induction is largely unknown, it seems universal that volatile-mediated priming targets defenses that are predominantly controlled by JA.

To date the physiological mechanism by which VOCs are able to prime plants is not understood. We also do not know the mechanism by which these sesquiterpenes prime and induce defenses when they are expressed in Arabidopsis. However, there are speculations that due to their lipophilic nature, VOCs generally might interact with the plasma membrane or any other hydrophobic structures or receptors (Godard et al., 2008; Heil et al., 2008; Zebelo et al., 2012). This may consequently lead to the activation of a sequence of early signaling events which include changes in cytosolic Ca^{2+} concentrations and downstream networks of kinases and phytohormones leading to the induction of defense response (Conrath et al., 2006; Maffei et al., 2007; Arimura & Maffei, 2010; Bricchi et al., 2010). Strong evidence supporting the involvement of calcium signaling during VOC priming was recently reported in Arabidopsis by Asai et al (2009). In conclusion, we obtained evidence that endogenous sesquiterpenes can act as signals to enhance defense responses of Arabidopsis against herbivores. Internal signaling by these compounds is not subjected to distance constraints and is not affected by environmental factors (wind, humidity, and temperature) which make volatile signaling unreliable. Thus,

future studies elucidating the molecular mechanisms that mediate volatile perception and signaling will be important steps to discern the ecological significance of this phenomenon.

2.4 Materials and Methods

2.4.1 Generation of the *35S::tps4* and *35S::tps5* transgenic Arabidopsis plants

The open reading frame (ORF) of the *tps4* (from inbred line B73) and *tps5* (from variety Delprim) genes were amplified by PCR from the pASK-IBA7 vector constructs (Köllner, 2004b) with the same primer set of forward: 5′-ATGGCGTCTCCTCCAGCA CATCG-3′and reverse: 5′-TCATTCGGGTATTGGCTCCACAAACAG-3′ and cloned independently into the pCR-TOPO vector (Invitrogen, Carlsbad, CA, USA) for sequencing. After sequence analysis, the 1,665 bp ORFs of *tps4* and *tps5* were re-amplified from the sequencing vector and cloned into the plant expression vector pB2GW7 using Gateway cloning technology between the *35S* promoter and the T35S terminator of the cauliflower mosaic virus. The resulting constructs were introduced into *Agrobacterium tumefaciens* strain GV3101, which was then used to transform *Arabidopsis thaliana* L. (ecotype Col-0) plants using the floral dip method supplemented with vacuum infiltration.

Since the constructs have a *bar* selectable marker gene that confers resistance to the herbicide Basta (Glufosinate), plants were screened for the transgene by applying Basta on soil-germinated young seedlings. To select for transgenic lines emitting the TPS4 and TPS5 sesquiterpenes, headspace volatiles were collected from detached leaves with SPME (solid phase micro extraction) and analyzed by GC-MS (see *Plant volatile collection and analysis* for the details). Three independent lines from each transformation event were selected for further biochemical, molecular, and insect physiological and behavioral assays.

2.4.2 Plant growth and insect rearing

Arabidopsis thaliana L. (ecotype Col-0) were grown in a commercially available potting soil in a climate controlled chamber with 150 µmol $m^{-2}s^{-1}$ photosynthetically

active radiation, a temperature cycle of 20/18 ^{o}C (day/night), a photoperiod of 8/16 h (light/dark), and 50 percent relative humidity. Five to six week-old Arabidopsis plants grown under such conditions were used for all biochemical, molecular, and insect feeding assays. Eggs of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) were obtained from Syngenta Crop Protection AG (Stein, Switzerland) and were incubated at 22^{o}C until hatching. Neonates were used for the long term no-choice feeding bioassays, while third instar larvae which were reared on a pinto bean based artificial diet (Perkins et al., 1973) were used for the behavioral and induction experiments.

2.4.3 Plant volatile collection and analysis

A dynamic headspace volatile collection system, which was installed in a climate controlled chamber (20/18 ^{o}C day/night temperature, 50 percent relative humidity, 150 µmol $m^{-2}s^{-1}$ photosynthetically active radiation, 8/16 h light/dark condition) was employed to collect volatiles from wild-type and transgenic Arabidopsis plants. In brief, five week-old Arabidopsis plants were placed independently in a 3 l glass cylinder (Schott, Mainz, Germany) which had air inlet and outlet valves on the lid. Charcoal-filtered air was allowed to enter to the cylinder with a flow rate of 1.2 l min^{-1} through Teflon tubing. The air exited the cylinder through a volatile collection trap, 150 X 6.4 mm glass tube (Analytical Research Systems, Gainesville, FL, USA) containing 30 mg Super Q adsorbent (Alltech, Deerfield, IL). All volatile collections were conducted during light-phase in a similar time frame for a period of 6 consecutive hours. The collected volatiles were eluted with 100 µl dichloromethane containing 5 ng $µl^{-1}$ nonyl acetate as internal standard.

Volatiles were then separated and identified by gas chromatograph (GC) coupled with mass spectrometer (MS) following the running conditions described in Fontana et al (2011). Compounds were identified by reference spectra in the Wiley and National Institute of Standards and Technology libraries and in the literature (Köllner et al., 2004b). For accurate quantification, compounds were analyzed by GC with a flame ionization detector (FID). Peak areas were compared with that of the internal standard, by applying a response factor of 1 for the internal standard and 0.74 for all the sesquiterpenes identified, as calculated according to the effective carbon number concept (Scanion and Willis, 1985).

2.4.4 Sesquiterpene extraction and quantification

To determine the internal pool of sesquiterpenes in the plant tissues, four week old Arabidopsis rosettes were harvested and ground to a fine powder with a pre-chilled mortar and pestle under liquid nitrogen. The powder (400-500 mg) was soaked in 1 ml pentane containing 1 ng μl^{-1} nonyl acetate as internal standard and gently agitated on a shaker at 180 rpm at 6 ^{o}C for 18 h. The sample was then clarified by passing the mixture through a glass column filled with glass wool and water-free sodium sulfate. The recovered and purified extract was then analyzed by GC-MS and quantified with GC-FID as described in section 2.4.3.

2.4.5 Insect feeding and orientation bioassays

No-choice bioassay: To determine *Spodoptera littoralis* larval performance on the sesquiterpene-overproducing transgenic lines, a no-choice feeding bioassay was conducted. In brief, six week-old Arabidopsis plants were infested with two newly hatched *Spodoptera littoralis* neonate larvae. In order to prevent larvae from escaping, each pot was enclosed in a perforated plastic bag and kept in a controlled chamber with 8/16 h light/dark photoperiod, 50 percent relative humidity, and a constant temperature of 22 ^{o}C. The larvae were allowed to feed without disturbance for five consecutive days and counts of surviving larvae and their weights were recorded daily starting from the fifth day and continued till pupation. To ensure sufficient food supply, larvae were transferred to new plants when approximately 70 percent of the rosette was consumed. Cumulative weight gains and percent survival were calculated. Days to pupation and pupa weight were also recorded and pupae were placed in a small plastic cup covered on the top with Whatman paper until the adult emerged. Adult emergence was monitored daily for a period of twenty days and date of emergence and adult weight at emergence were recorded.

Multiple-choice bioassay: To test the larval feeding preference for the transgenic or wild-type Arabidopsis plants, a relatively short-term multiple-choice bioassay was conducted. Briefly, a 30 x 30 x 30 cm choice arena was built which consisted of a 30 x 30 cm acrylic platform in the middle of the arena. The platform has six holes (5 cm diameter each) arranged at regular distances from each other in a circular manner and each hole was positioned 8 cm away from the center of the platform. Rosette leaves of three

transgenic and three wild-type plants were exposed on the choice platform through the holes in an alternate manner. Ten to twenty third instar *Spodoptera littoralis* larvae reared on artificial diet were released in the middle of the choice platform and allowed to feed on their preferred plant genotype for a period of 18 h. Damaged leaves from each individual plant were collected and scanned on a Bookeye scanner (Image Access, Wuppertal, Germany) together with a 4 cm^{-2} reference area. The leaf area consumed on each plant was calculated using SigmaScan Pro5 software based on the pixel values of the reference and consumed leaf area. Percent leaf area consumed was calculated and used as an index of larva feeding preference. The Wilcoxon matched-pairs test (SPSS, v. 15) was used to compare the amounts of leaf area consumed on wild-type and transgenic lines. Three to five independent choice experiments were conducted on each transgenic line against the wild-type Col-0.

Orientation assay: In order to test the effect of sesquiterpene volatiles on the feeding orientation of *Spodoptera littoralis* larvae, a short-term orientation assay which lasted for 1 h was conducted. Briefly, a modified six arm multiple-choice arena was built (70 x 70 x 70 cm). Like the above arena, it was equipped with a choice-platform in the middle with six alternately arranged holes having a distance of 20 cm from the center of the platform. Three transgenic and three wild-type plants were alternately positioned exactly underneath each hole, but without exposing the rosette leaves on the choice platform. Unlike the previous arena, no visual or gustatory cues from the plants were available to the larvae; only olfactory cues were supplied. Groups of twenty larvae were released in the middle of the platform. After 1 h, the larvae trapped in each hole were collected and counted.

2.4.6 Extraction and quantification of glucosinolates

To compare the glucosinolate concentrations in the transgenic and wild type Arabidopsis plants, intact and herbivore damaged rosette leaves were harvested, freeze-dried, and ground to a fine powder using a paint shaker. Approximately 20 mg of the powder was extracted with 1 ml of 80 percent (v/v) methanol containing 0.05 mM internal standard (4-hydroxybenzylglucosinolate). The samples were then centrifuged at 8000 rpm for 10 min and the supernatant was loaded on a 96-well format filter plate (Nunc, Langenselbold, Germany) pre-filled with DEAE-Sephadex. Wells were washed with 0.5 ml 80 percent (v/v) methanol, 0.5 ml water, and 0.5 ml 0.02 mM MES buffer

(pH 5.2) respectively. To each well, 25 µl of sulfatase solution was directly applied on the top of the filter. After overnight incubation at room temperature, desulfo-glucosinolates were eluted with 0.5 ml water.

The samples were analyzed with high performance liquid chromatography (HPLC) on an Agilent HP1100 series instrument equipped with a C-18 reversed phase column (LiChrospher RP18ec, 250 X 4.6 mm, 5 µm particle size). An injection volume of 50 µl extract was run in a solvent gradient of water (Solvent A) and acetonitrile (solvent B) at a flow rate of 1 ml min^{-1} at 25 ^{o}C. Desulfoglucosinolates were identified based on comparison of retention times and UV absorption spectra with the internal standard. Results are given as µmol g^{-1} dry weight calculated from the peak area of 229 nm relative to the peak area of the internal standard using the relative response factors of 2.0 for aliphatic and 0.5 for indole glucosinolates.

2.4.7 Extraction of RNA, cDNA synthesis and qRT-PCR

Total RNA was extracted from 100 mg fresh Arabidopsis tissue using the Qiagen RNeasy Plant Mini kit (Hilden, Germany) according to the manufacturer's instructions. To remove residual genomic DNA, the extracts were treated with RNAse-free DNAse (Qiagen, Hilden, Germany). RNA quality was measured with an Agilent 2100 Bioanalyzer (Waldbronn, Germany) and quantified spectrophotometrically with Nanodrop 2000C (Thermo Fisher, Wilmington, DE, USA). For cDNA synthesis, Superscript III reverse polymerase (Invitrogen, Carlsbad, USA) was used following the manufacturer's instruction but with 5 µg total RNA in 20 µl reaction volume. The qRT-PCR was performed on 1:50 diluted cDNA templates in a Stratagene Mx3000P real-time PCR machine (La Jolla, CA, USA) using SYBR Green-based (Applied Biosystems, Darmstat, Germany) detection of dsDNA synthesis. The linear range of template concentration to threshold cycle value (C_t) was determined by performing a series of six-fold dilutions using cDNA from three independent RNA extractions. Primer efficiencies were calculated using the standard curve method (Pfaffl, 2001). All primers were designed using Beacon primer design software (Primer Biosoft, Palo Alto, USA) and ordered to be HPLC-purified (Invitrogen). The relative transcript level was calculated using *Actin* as normalizer gene and untreated wild-type Col-0 as calibrator. All primer pairs used for the qRT-PCR are listed in supplementary materials section (Table S9.1.1).

2.4.8 Radial diffusion assay for TPI activity determination

Trypsin proteinase inhibitor (TPI) activity was determined using a radial diffusion assay protocol described by Jongsma et al. (1994). In brief, the method is based on the radial diffusion of a protein extract from a central well through a plant agar (1.8 percent w/v in 0.1 M Tris Cl buffer, pH 7.6) containing trypsin proteinase with a final concentration of 0.001 mg ml^{-1}. The agar was poured in a 12 cm x 12 cm Petri dish placed on a level surface and allowed to solidify at 4^{o}C. Holes were punched out with a 4 mm diameter cork-borer 1.5 cm apart. Extracted samples (approximately 40 µl) were added to the holes and allowed to diffuse through the gel overnight. After 18 h incubation at 4 ^{o}C the agar gel was stained with a solution consisting of 48 mg Fast Blue salt (*0*-dianisidine) in 80 ml 0.1 M Tris Cl, pH 7.6, mixed with 24 mg *N*-acetyl-DL-phenylalanine-naphthyl ester in 20 ml DMF (*N,N*-dimethylformamide) at 37 ^{o}C for 1 hr and then rinsed with tap water. Circular zones containing inhibitor-proteinase complexes remained colorless, while the region containing only proteinase showed a bright pink-purple color. TPI activity was quantified by measuring the diameter of the clear zones around each well with a caliper while the plate was placed on a transilluminator. A reference curve related to the diameter of the colorless zone to the logarithm of the PI concentration was prepared with the soybean proteinase inhibitor standard (Sigma). Soluble protein content of each extract was determined in ELISA plates by the Bradford assay (Bradford, 1976) using the TECAN GENios plate reader. Levels of PIs are reported in nanomoles of inhibited proteinase molecules per milligram soluble leaf protein.

2.4.9 Phytohormone extraction and analysis

For the herbivore-induced quantification of jasmonates, five week-old Arabidopsis plants were infested with three third instar *Spodoptera littoralis* larvae and allowed to feed for a period of 24 h. Herbivore-damaged rosettes were then harvested, flash-frozen in liquid nitrogen and stored at minus 80 ^{o}C until use. For wound-induced short term jasmonate analysis, plants of the same age were mechanically wounded by a pattern wheel along the midvein and treated with 1:5 diluted S. *littoralis* larvae oral secretion on the wounded site. To the wounded controls only deionized water was applied. Samples were then harvested at two time points after the treatments (30 and 60 min after the treatment). All samples were ground into fine powder with pre-cooled mortar and pestle and 200 mg of the powdered sample was transferred into a 2 ml

Eppendorf tube containing 500 mg FastPrep matrix (BIO 101, Vista, USA). To all the samples, 0.5 ml extraction buffer (pure MeOH) spiked with 10 ng D_2-JA and 2 ng $^{13}C_6$-JA-Ile as internal standards was added and homogenized in a paint shaker by reciprocal shaking for 5 min. Then the samples were vigorously vortexed for 5 min and centrifuged at 13,000 rpm for 20 min at 4 ^{o}C. Then 300 µl of the supernatant was collected in HPLC vials. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies, Boeblingen, Germany). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 x 4.6mm, 1.8µm, Agilent, Waldbronn, Germany). Formic acid (0.05 percent) in water and acetonitrile were employed as mobile phases A and B respectively. An API 3200 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a Turbospray ion source was operated in the negative ionization mode. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion-product ion: m/z 209.1-59.0 for jasmonic acid; m/z 213.1-56.0 for 9,10-D_2-dihydrojasmonic acid; m/z 322.2-130.1 for jasmonic acid-isoleucine conjugates; m/z 328.2-136.1 for jasmonic acid-$^{13}C_6$-isoleucine conjugate.

3. Research Chapter II

Transgenic Arabidopsis plants expressing the maize TPS10 and TPS23 sesquiterpenes are better protected from ozone-induced damage*

Abstract

Plants produce and emit a diverse array of volatile organic compounds that play important roles in plant-insect and plant-plant interactions. Apart from their role in plant adaptations to biotic stresses, terpenoid-based volatiles like isoprene and monoterpenes take part in plant defense reactions against ozone induced oxidative stresses. Sesquiterpenes share a common biosynthetic origin and physiochemical properties with isoprene and monoterpenes, yet their biological role in ozone-stressed plants is largely unknown. In this study we investigated whether transgenic Arabidopsis plants overexpressing genes of sesquiterpene biosynthesis are better protected from ozone induced injury. We transformed Arabidopsis with two maize sesquiterpene synthase genes, *Zm*TPS10 and *Zm*TPS23, whose sesquiterpene products are known to react highly with ozone. We showed that Arabidopsis plants overproducing the sesquiterpenes were better protected from ozone damage and displayed less visible injury, reduced ion leakages, maintain their photosynthetic efficiency and generate lower oxidative stress compounds compared to the wild type. The protective role of the sesquiterpenes against ozone damage could be attributed to their ozone quenching abilities at the leaf boundary layer. We showed a 67 to 79 percent degradation of ozone in the headspace of the transgenic plants which were fumigated with a 300 ppb preset ozone concentration in exposure chambers. This degree of ozone cleansing by the sesquiterpenes can presumably reduce the ozone uptake and subsequent oxidative damage to plant cells. This hypothesis was further supported by an *in vitro* fumigation assay with authentic (*E)*-β-caryophyllene and (*E)*-β-farnesene standards in which 59 to 70 percent of the ozone present in fumigation chambers was degraded by the respective sesquiterpene standards. Sesquiterpenes may therefore protect plants against ozone damage by a mechanism similar to that of isoprene. However, since sesquiterpenes are emitted from more plant species than isoprene, these compounds might have a greater ecological relevance for adaptation to ozone stress.

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3.1 Introduction

Plants synthesize and emit a wide spectrum of volatile organic compounds (VOCs), with terpenoids being one of the dominant classes. Since a significant proportion of the net carbon assimilated by photosynthesis is emitted as VOC emission (Sharkey & Yeh, 2001), it was proposed that plants must gain some benefits from its synthesis (Lerdau, 2007). Due to their volatility, VOCs can transfer chemical messages away from their site of synthesis. For example, they function as chemical messengers to convey signals to parasitoids and predators about the location of their respective prey (Rasmann et al., 2005; Schnee et al., 2006; Fontana et al., 2011). Volatiles also mediate plant-to-plant communications to prime defenses against insect pests (Engelberth et al., 2007; Ton et al., 2007; Frost et al., 2008).

Apart from their role in plant adaptations to biotic stresses, VOCs have been shown to take part in plant defenses against thermal and ozone stresses. Tropospheric ozone is a major secondary air pollutant produced by a series of photochemical reactions from primary emissions of nitrogen oxides (NOx) and VOCs (Atkinson & Arey, 2003). Hence, VOCs may have contradictory functions to plants: in the presence of other anthropogenic pollutants in the atmosphere, VOCs aggravate the negative effect of ozone by enhancing its synthesis; whereas in the absence of these pollutants, VOCs purge ozone and safeguard plants. The global background concentration of this pollutant has been rising since the Industrial Revolution and was widely recognized as a problem affecting both natural forest communities and crop species (Ashmore & Marshall, 1999). Since ozone entry through the leaf cuticle is negligible (Kerstiens & Lendzian, 1989), the flux of ozone into the apoplastic space is largely controlled by the rate of stomatal gas exchange (Vahisalu et al., 2008). After entering a leaf via stomata, ozone rapidly breaks down in the apoplastic fluid and generates several reactive oxygen species (ROS) (Mahalingam et al., 2006). These free radicals and their products react with proteins, DNA and membrane lipids to cause reduced photosynthesis efficiency, electrolyte leakage and accelerated senescence (Sharma & Davis, 1997).

Other than its direct negative impact on the physiology of plants, recent studies have shown that ozone can also indirectly affect the ecological functions of plant volatiles by degrading them. For example, ozone was shown to degrade several herbivore-induced plant volatiles ultimately impeding plant-to-plant communications by reducing the

effective communication distance (Blande et al., 2010). A modeling study by McFrederick et al (2008) suggested that degradation of floral scents by ozone episodes might lead to reduced searching and foraging efficiency of pollinators. In contrast, recent work by Pinto et al (2007ab) showed that although ozone degrades common herbivore-induced plant volatiles, the ability of predators and parasitoids to find plants is not lost. This may indicate the presence of other volatile cues that are less vulnerable to ozone.

A number of studies have been performed to understand the genetic basis of the vastly different ozone sensitivity displayed among plant species. The majority of these investigations focused on a correlation of stomatal conductance, photosynthetic rates, phytohormone levels and levels of antioxidant compounds and enzymes (Koch et al., 2000; Tamaoki et al., 2003; Brosche et al., 2010; Guidi et al., 2010). However, recent advances in this field revealed that plant volatile profiles also play an indispensable role in resistance to ozone stress (Heiden et al., 1999; Loreto & Velikova, 2001; Loreto et al., 2004). Most of these investigations, however, focused only on isoprene (C_5H_8).

Isoprene has been demonstrated to reduce ozone damage and to quench ozone and ROS. For instance, when the natural capacity of *Phragmites australis* plants to synthesize and emit isoprene was inhibited by fosmidomycin, the plants natural ability to resist ozone is compromised (Loreto & Velikova, 2001). Another good example demonstrating this biological function of isoprene is the overexpression of isoprene synthase genes in ozone sensitive, non-isoprene emitting plants which rendered the plants resistance to ozone stress (Vickers et al., 2009). Ozone resistance manifested itself by decreased foliar damage, increased photosynthetic efficiency and reduced accumulation of reactive oxygen species. The mechanisms by which isoprene mediates ozone resistance were proposed to be the direct ozone quenching of isoprene at the leaf surface (Loreto et al., 2001) and the scavenging of ROS generated after ozone enters in to the plant cell (Affek & Yakir, 2002; Velikova et al., 2004). Monoterpenes ($C_{10}H_{16}$) have a similar antioxidant function in ozone stressed *Quercus ilex* plants (Loreto et al., 2004). Unlike other oak species, *Quercus ilex* does not naturally produce isoprene, but it emits monoterpenes dominated by α-pinene, β-pinene and sabinene. When the monoterpene production of leaves was blocked by fosmidomycin, ozone reduced the photosynthetic efficiency and increased the production of hydrogen peroxide (H_2O_2) and malonyldialdehyde (MDA).

Although sesquiterpenes ($C_{15}H_{24}$) share a common biosynthetic origin and physiochemical properties with isoprene and monoterpenes, their biological role in ozone stressed plants is largely unknown. However, sesquiterpenes might be important in plant adaptation to ozone stress due to their high reactivity towards ozone (Griffin et al., 1999). It has been recently estimated that the mean daytime ozone loss in a central Amazon canopy due to gas-phase reaction with sesquiterpenes to be between 7- 28 percent (Jardine et al., 2011). Thus, by acting as an effective ozone sink within plant canopies, sesquiterpenes may significantly reduce the harmful uptake of ozone and the associated oxidative damage. Additionally, ozone has been reported to induce the emission of mono- and sesquiterpenes in exposed tobacco and scots pine plants (Heiden et al., 1999). Interestingly, the ozone-resistant tobacco cultivar Bel B was able to rapidly release large amount of sesquiterpenes dominated by β-caryophyllene and valencene. However, the susceptible cultivar, Bel W3, could emit these volatiles only 24 h after ozone exposure. This difference might be important since the first few minutes of ozone uptake and the subsequent defensive measures taken by plants determine the degree of damage caused by ozone.

In this chapter, we studied the roles of sesquiterpene blends encoded by two maize sesquiterpene synthases, *ZmTPS10* and *ZmTPS23,* in ozone-stressed plants by genetically transforming Arabidopsis with the respective genes. *ZmTPS10* produces two major sesquiterpene volatiles *(E)*-β-farnesene and *(E)*-α-bergamotene; whereas *ZmTPS23* generates *(E)*-β-caryophyllene as a major product and α-humulene as trace product. In order to determine their defense response, plants were exposed to either acute or chronic levels of ozone and assessed for visible injuries, accumulation of reactive oxygen species, membrane integrity, photosynthetic capacity, phytohormone levels, and stress gene expression. To determine any protective role by the sesquiterpenes at the leaf boundary layer, we conducted ozone quenching assays with authentic *(E)*-β-caryophyllene and *(E)*-β-farnesene standards in test chambers without plants. This study highlights the potentials of transgenic Arabidopsis plants to investigate the ecological relevance of other related volatiles during ozone stress.

3.2 Results

3.2.1 Generation of transgenic Arabidopsis plants producing the test sesquiterpenes

Arabidopsis thaliana ecotype Col-0 was transformed with the maize sesquiterpene synthase genes *tps10* and *tps23* which were placed under the control of the constitutive CaMV *35S* promoter linked to the phosphinothricin acetyltransferase (*bar)* selectable marker gene to facilitate the identification of putative transgenic plants (Fig. 3.1a). In a preliminary screening for the ozone inducibility of sesquiterpene biosynthetic genes in maize, we found only these two genes to be highly induced by ozone exposure (Fig. S9.2.1). Transgenic Arabidopsis plants with the *35S::tps10* construct produced *(E)*-α-bergamotene and *(E)*-β-farnesene, whereas those transformed with the *35S::tps23* constructs produced (*E)*-β-caryophyllene and trace levels of *α*-humulene from their rosette leaves. In wild-type Col-0 plants none of these sesquiterpene types were detected at the rosette stage (Fig. 3.1b) although wild-type Col-0 can emit *(E)*-β-caryophyllene from its flower stigmas during flowering (Huang et al., 2011). The combined emission rate of these sesquiterpenes, *(E)*-β-caryophyllene and α-humulene, in the *35S::tps23* transgenic lines was between 36 and 66 ng h^{-1} g^{-1} FW. A similar emission rate (between 42 and 56 ng h^{-1} g^{-1} FW) was observed for *(E)*-α-bergamotene and *(E)*-β-farnesene in the *35S::tps10* transgenic lines (Fig. 3.1c). Two independently transformed lines of each construct were selected for further analysis in response to ozone fumigation. Additionally, the same sesquiterpenes were also tested in a relatively ozone susceptible Arabidopsis background by genetically transforming the respective genes in Ws-2 Arabidopsis ecotype. A genetically modified maize line with the oregano *(E)*-β-caryophyllene synthase gene has been also included in this ozone fumigation study. All results generated based on these two latter plant systems are reported in the supplementary materials section (9.2).

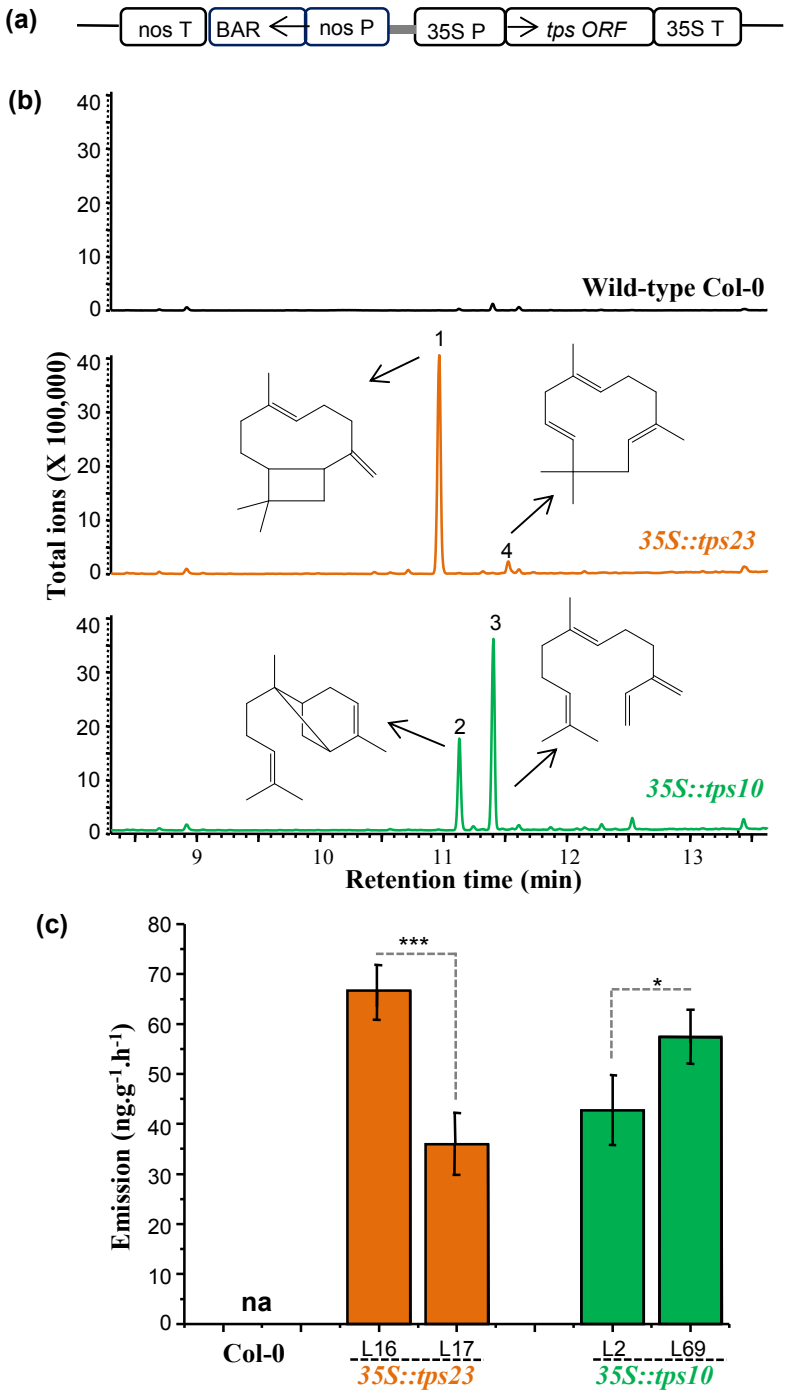

Fig. 3.1: Engineering Arabidopsis plants overexpressing maize sesquiterpene synthase genes. **(a)** *35S::tps* construct scheme used to transform Arabidopsis. *Nos T*-nopaline synthase terminator, *nos P*-nopaline synthase promoter, BAR-Basta resistance gene, *tps* ORF-terpene synthase open reading frame. **(b)** Headspace volatile collections from rosette leaves of Arabidopsis and subsequent analysis with GC-MS. The sesquiterpenes identified in the transgenic plants are as follows: 1. *(E)*-β-caryophyllene; 2. *(E)*-α-bergamotene; 3. (*E*)-β-farnesene; and 4. α-humulene. **(c)** Quantification of the products with GC-FID. Asterisks indicate statistical differences on the emission rates between lines in the same gene transformation (student's t-test, $P \leq 0.05$).

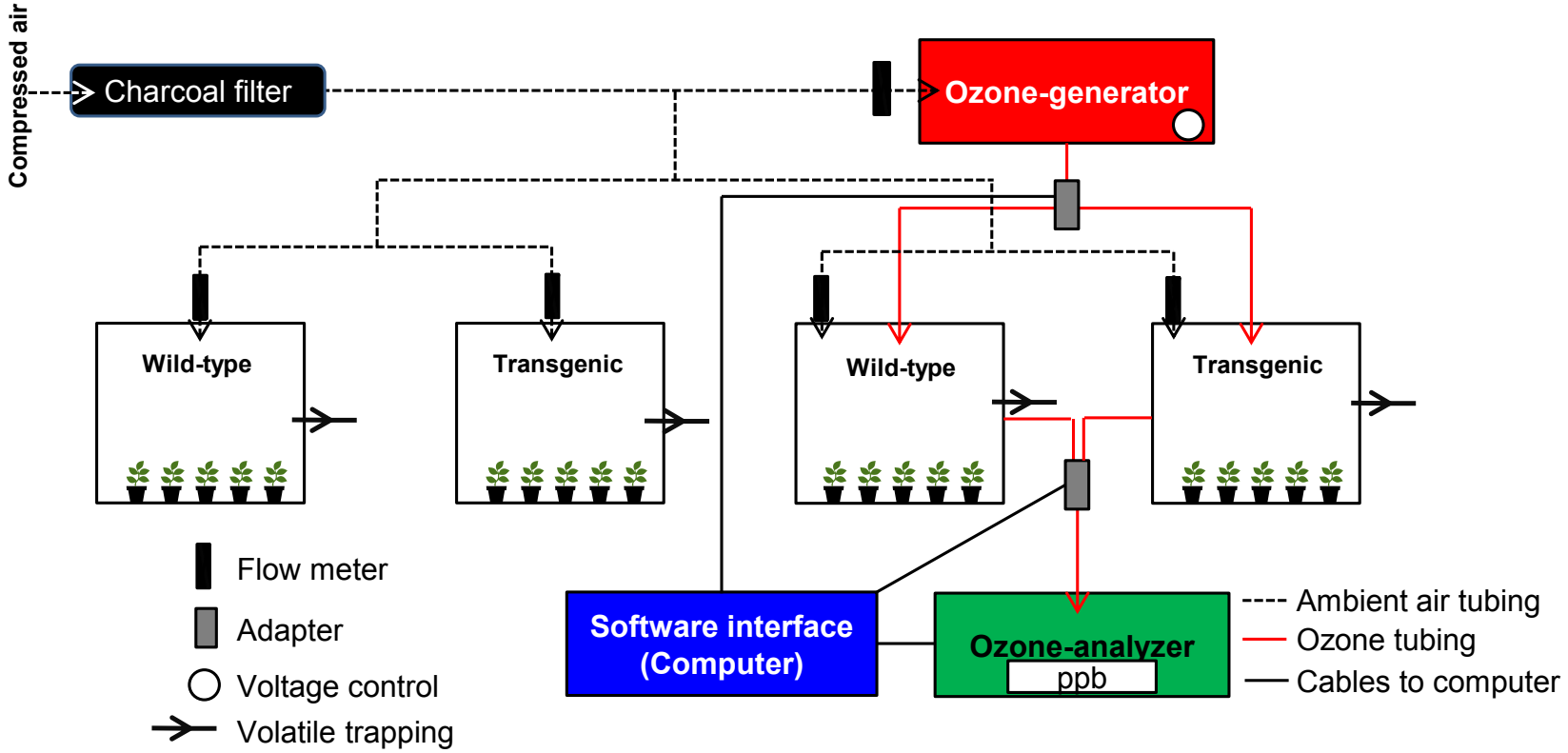

Fig. 3.2: Schematic diagram of the ozone exposure experimental setup. Wild-type and sesquiterpene expressing transgenic plants were fumigated in separate exposure boxes. All the system was put in a dedicated climate chamber. The required ozone concentrations in the exposure boxes were adjusted with a combination of options including dilution air flow rate adjustment, voltage control on the ozone generator, ozone discharge-pause timing intervals to the exposure boxes controlled by LabVIEW software (National Instruments, Munich).

3.2.2 Sesquiterpene producing transgenic plants are resistant to ozone-induced damage

All ozone exposures were carried out in fumigation boxes built in a dedicated climate chamber (Fig. 3.2). In order to exclude the protective effect of volatilized sesquiterpenes on neighboring non-emitters, wild-type and sesquiterpene emitting transgenic plants were exposed to ozone in separate fumigation boxes.

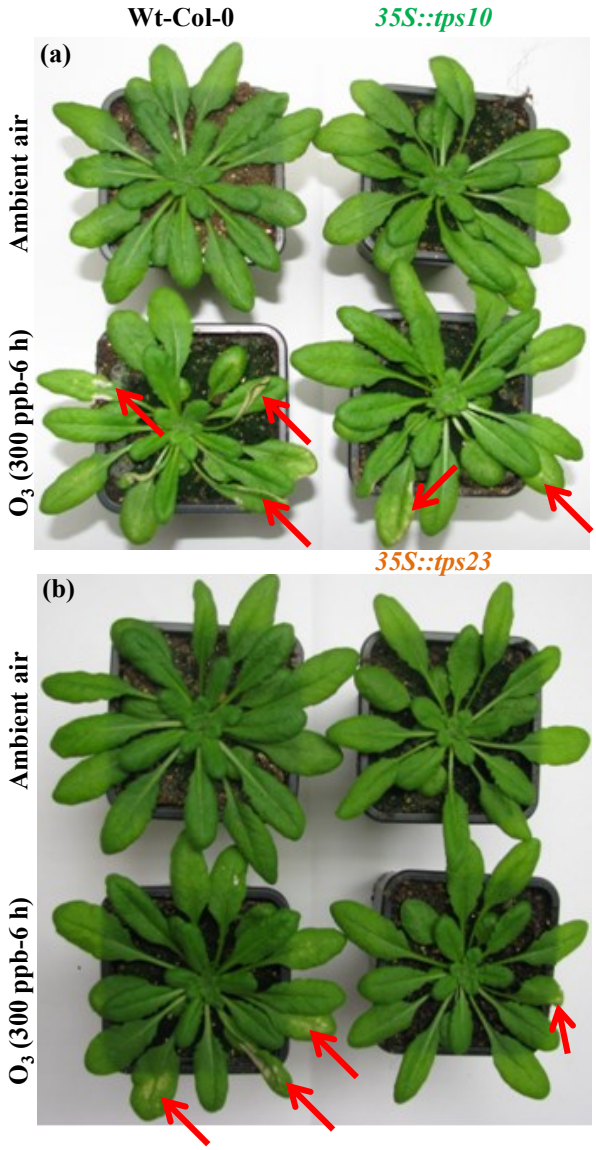

Fig. 3.3: Ozone-induced visible injuries on wild-type and sesquiterpene producing transgenic Arabidopsis plants. Plants were fumigated with 300 ppb ozone for 6 h and photographs were taken after three days post fumigation. In **(a)** wild-type Col-0 and the maize TPS10 sesquiterpene expressing Arabidopsis plants were compared; while in **(b)** wild-type Col-0 and transgenic Arabidopsis plants expressing the maize TPS23 sesquiterpenes were compared. Red arrows indicate visible damages on leaves and necrotic and chlorotic leaf areas are designated as n and c respectively.

Visible injury of leaves fumigated with acute level of ozone (300 ppb for 6 h) was screened immediately after and 72 h post fumigation in wild-type and transgenic plants. The plants developed lesions on their leaves immediately after the end of ozone exposure and these lesions turned into clearly visible necrotic or chlorotic areas three days post fumigation (Fig. 3.3). Interestingly, these necrotic and chlorotic leaf areas were less pronounced in the sesquiterpene-overproducing lines compared to the wild-type. In order to estimate the degree of leaf damage quantitatively, we measured ion leakage as indicator of leaf injury after a single ozone exposure at 300 ppb for 6 h. Ozone-induced ion leakage increased 2-fold in wild-type Col-0 plants whereas no significant increase in ion leakage was observed in both the 35S::*tps10* and 35S::*tps23* transgenic lines (Fig. 3.4). After acute ozone exposure (300 ppb, 6 h), a similar reduction of ion leakage was also observed in the (*E*)-β-caryophyllene overproducing Hi-II maize line (Fig. S9.2.2) and transgenic Ws-2 Arabidopsis ecotype transformed with the *35S::tps10* and *35S::tps23* constructs (Fig. S9.2.6).

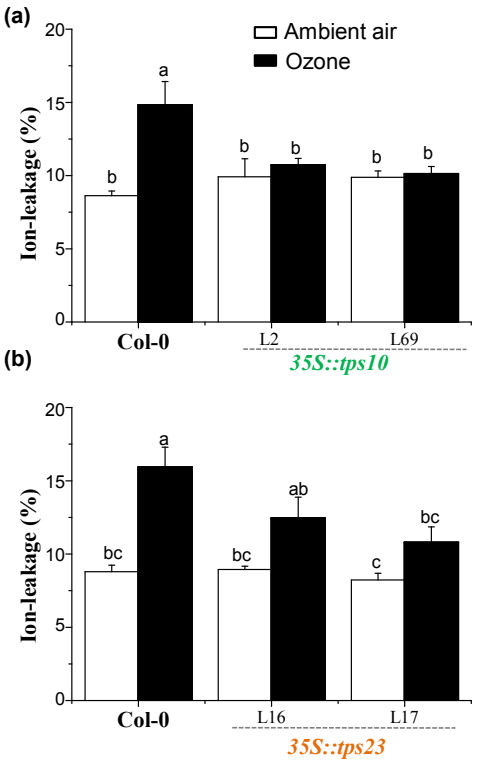

Fig. 3.4: Quantification of ozone-induced leaf damage on the basis of relative total ion leakages from **(a)** the *35S::tps10* and **(b)** *35S::tps23* leaves. Leaf samples were collected (14 mm Ø) after plants were fumigated continuously with ozone at a concentration of 300 ppb for 6 h and floated on deionized water for 3 h. Conductivity was measured and ion-leakage was expressed in terms of percentage based on the conductivity recorded after the samples were boiled to get the total electrolyte content. The graphs show mean ± SE of 5 replicates. Bars with different letters represent significant differences at $\alpha = 0.05$ after ANOVA followed by Tukey HSD analysis.

3.2.3 Short-term ozone exposure did not affect the photosynthetic response of the transgenic lines

To study the effect of acute short-term ozone exposure on the photosynthetic response of wild-type and sesquiterpene-emitting plants, gas exchanges was measured in ambient air and after the plants were exposed to 200 ppb ozone for 2, 3, and 6 h. In ambient air, both CO_2 assimilation rate and stomatal conductance appeared to be larger in the wild-type Col-0 plants compared with the *35S::tps10* and *35S::tps23* transgenic lines, but this difference was not significant (Fig. 3.5a). After 2 h ozone exposure, the wild-type Col-0 and the *35S::tps10* transgenic lines reduced their original ambient level photosynthetic rates by 11 percent, while only a 5 percent decline was observed in the *35S::tps23* lines. However, these declines in photosynthetic response were not significant compared to their respective ambient level responses. Interestingly, after 3 h ozone exposure, the photosynthetic efficiency of wild-type was reduced by 32 percent and this reduction was statistically significant ($F_{3,12}$ = 5.41, p = 0.01). Whereas, both the *35S::tps10* and the *35S::tps23* transgenic lines did not change their photosynthetic rate significantly. Only after 6 h ozone exposure did the *35S::tps10* lines reduced their photosynthetic efficiency significantly ($F_{3,18}$ = 3.07, p = 0.05). However, in the *(E)*-β-caryophyllene emitting *35S::tps23* lines, 6 h ozone exposure did not affect their photosynthetic response significantly ($F_{3,12}$=1.73, p=0.21). Additionally, we did not observe any significant reduction in stomatal conductance in all the ozone exposure times and there was not either any difference in all the Arabidopsis genotypes tested (Fig. 3.5b). An acute ozone exposure (300 ppb, 6 h) on wild-type and *(E)*-β-caryophyllene emitting transgenic Hi-II maize lines resulted in reduced photosynthetic efficiency and stomatal conductance in the wild-type Hi-II plants measured after a recovery time of 18 h (Fig. S9.2.4).

3.2.4 Transgenic lines accumulated less H_2O_2, MDA and displayed reduced cell death after ozone exposure

Exposure to 300 ppb ozone for 6 h resulted in an increase in the H_2O_2 content of the wild-type Col-0 plants. However, we did not see any ozone-induced accumulation of H_2O_2 for the sesquiterpene overproducing lines (Fig. 3.6a). In some transgenic lines like *35S::tps10* L69, the ambient level of H_2O_2 was even found to be higher than the ozone-induced level. Considering the technical difficulties in quantifying tissue H_2O_2 extracts

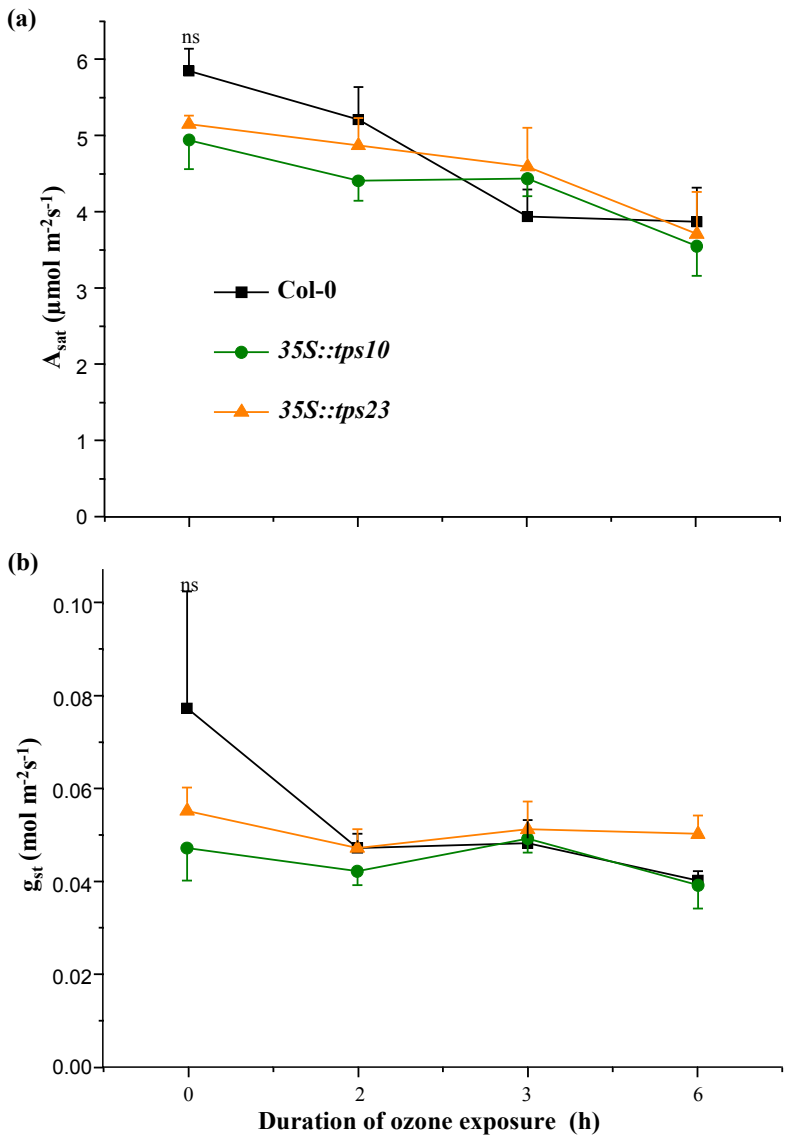

Fig. 3.5: CO_2 assimilation rate (A_{sat}) **(a)** and stomatal conductance (g_{st}) **(b)** in wild-type Col-0 and the TPS10 and TPS23 sesquiterpene overexpressing transgenic Arabidopsis lines. Measurements were taken immediately after acute (200 ppb) ozone exposure for 2, 3, and 6 hours. Ambient air exposed control measurements were represented by the 0 h reading. The infrared gas analysis was conducted at 400 µmol m^{-2} s^{-1} PAR; CO_{2Ref} of 380 µmol; T^0_{leaf} of 24 °C. ANOVA was conducted using the R statistical package on n = 4-6 replicates and significant response patterns within each group are reported in the text.

accurately (Queval et al., 2008), we did a more qualitative and fast detection of H_2O_2 accumulations using DAB staining. As a result, we found that plants tend to accumulate more H_2O_2 after acute level ozone fumigation (300 ppb, 6 h) which was shown by having deep brown coloration (Fig. 3.6c). The degree of brown coloration was more pronounced in the wild-type Col-0 plants. A similar effect, though to a lower extent, was observed in the level of MDA, an indicator of lipid peroxidation, in the wild-type Col-0 plants (Fig.

3.6b). Ozone-induced cell death was also much less pronounced in the sesquiterpene overproducing lines which were shown by having less blue coloration after trypan blue staining (Fig. 3.6c). The same patterns of effects were observed in *(E)*-β-caryophyllene overproducing transgenic Hi-II maize lines (Fig. S9.2.3).

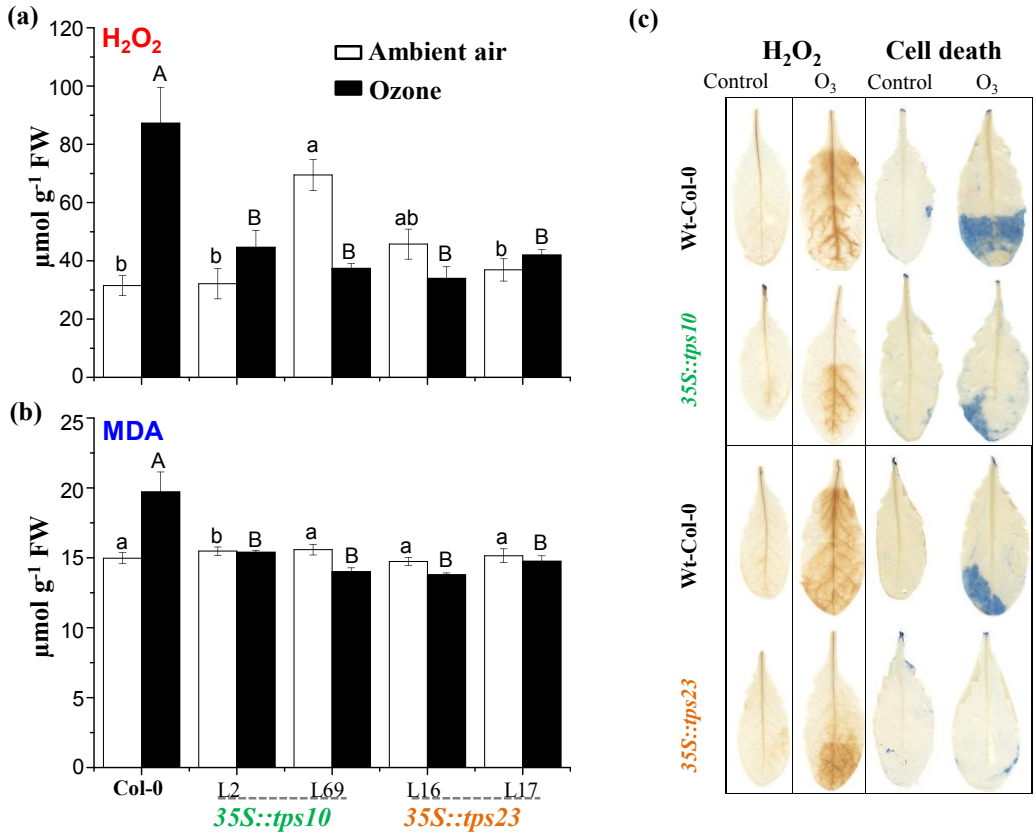

Fig. 3.6: Effect of acute ozone exposure (300 ppb, 6 h) on **(a)** H_2O_2 and **(b)** Malondialdehyde (MDA) accumulation. Histochemical staining of Arabidopsis leaves for qualitative detection of H_2O_2 and cell death **(c)**. For H_2O_2 detection leaves were stained with DAB solution (3,3'-Diaminobenzidine) and for cell death localization leaves were stained with lactophenol-trypan blue solution. Deep brown coloration represents H_2O_2 accumulation and dead cells are represented by blue coloration. Bars with different letters represent significant differences at α = 0.05, n = 6-9 replicates after ANOVA followed by Tukey HSD analysis.

3.2.5 Ozone-induced hormone accumulation and gene expression in wild-type and transgenic lines

To determine if there is any differential accumulation of stress hormones or gene expression in wild-type and sesquiterpene-overproducing plants, we measured the concentrations of common phytohormones involved in ozone induced signaling and

analyzed the expression of ozone responsive marker genes after short-term ozone exposures. Continuous ozone exposure at 300 ppb for 6 h caused a rapid induction of SA in all Arabidopsis genotypes, though the amount was two-fold larger in the wild-type Col-0 plants compared to both the TPS10 and TPS23 sesquiterpene producing lines (Fig. 3.7a). Interestingly, the *35S::tps23 (E)*-β-caryophyllene producing transgenic lines accumulated lower baseline concentration of SA compared to the wild-type and the *35S::tps10* transgenic lines. Unlike SA, the response of JA to ozone exposure was not dramatic and it was only slightly induced in wild-type plants. In the transgenic lines, JA

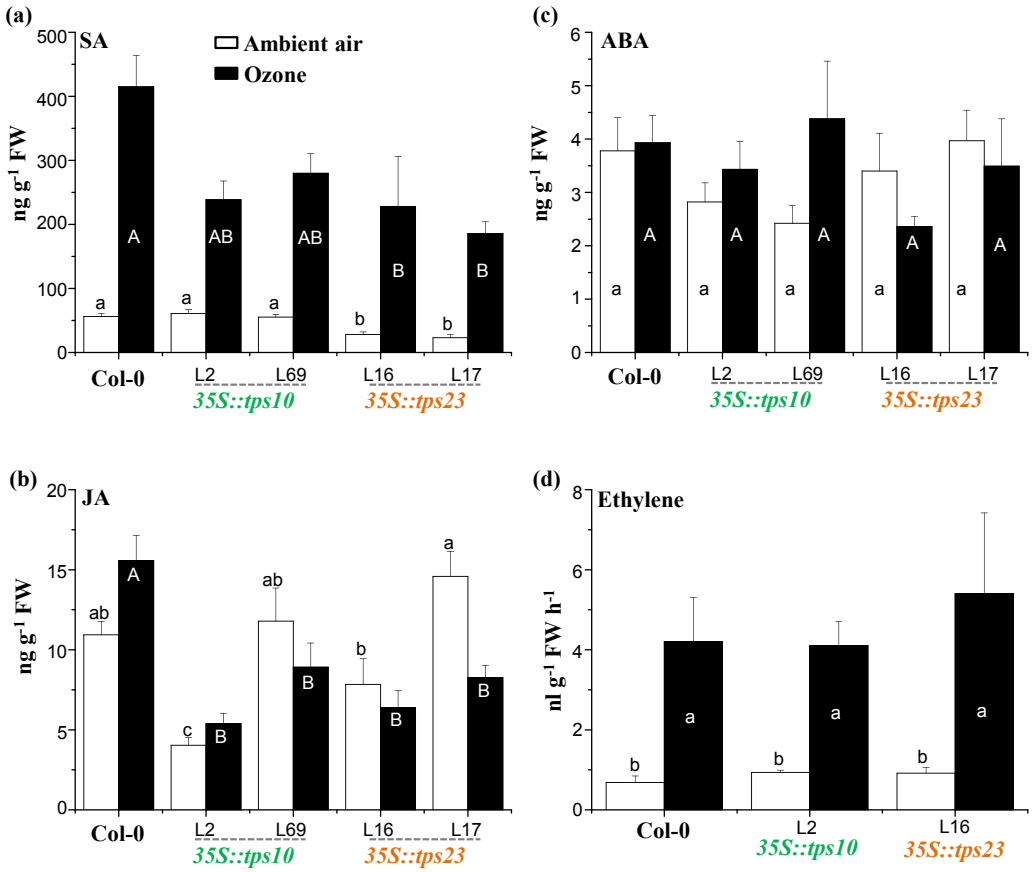

Fig. 3.7: Ozone-induced phytohormone responses of wild-type and sesquiterpene overexpressing Arabidopsis lines. Plants were exposed to ambient air (open bars) or 300 ppb ozone (black bars) for 6 h. **(a)** SA, **(b)** JA, and **(c)** ABA were analyzed in the same sample (n = 6-12); whereas, **(c)** ethylene was analyzed in a separate experiment but with similar ozone exposure conditions (n = 3). Bars with different letters represent significant differences at α = 0.05 after ANOVA followed by Tukey HSD analysis.

seems either unresponsive to ozone exposure or slightly decreased in some of the transgenic lines (Fig. 3.7b). ABA, which is assumed to be induced by ozone exposure, was found unchanged in all the genotypes after ozone exposure (Fig. 3.7c). In some Arabidopsis accessions, genetic variation to ozone sensitivity is based on differences in

their ethylene biosynthesis. In order to understand whether this is true for our system, we analyzed ozone-induced ethylene emissions in wild-type and sesquiterpene overproducing transgenic Col-0 plants. Ethylene emission was increased four-fold from the ambient air controls in all the genotypes but there was not any significant difference between the wild-type and the sesquiterpene overproducing lines (Fig. 3.7d).

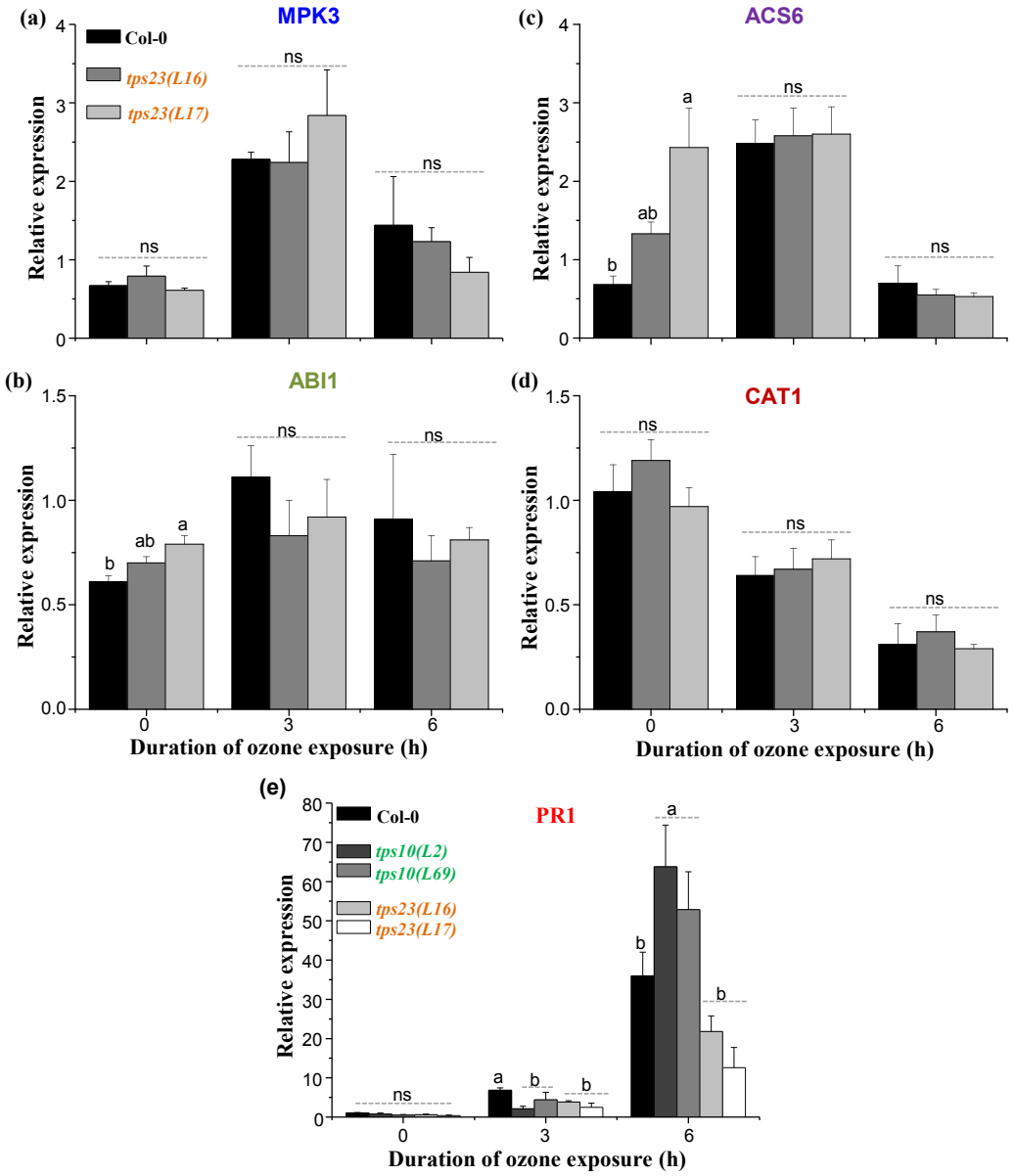

Fig. 3.8: Analysis of ozone-induced gene expressions in wild-type and sesquiterpene overproducing transgenic Arabidopsis lines. Plants were exposed to ambient air for 6 h or 300 ppb ozone for 3 h or 6 h. Samples were collected immediately after the end of the experiment for RNA extraction. Gene expression was conducted on three biological replicates and normalized by ACTIN expression. Bars with different letters represent significant differences after ANOVA followed by Tukey HSD test at $\alpha = 0.05$. ns = stands for nonsignificant differences.

We also analyzed the expression of selected marker genes involved in the ozone-induced defense signaling and proteins involved in the defense responses. One of the early activated oxidative-stress related genes, the mitogen-activated protein kinase3 (*AtMPK3*) was two-fold induced in all the genotypes tested after the first 3 h ozone exposure (Fig. 3.8a). The *ABIl* gene which is assumed to be involved in regulating ozone-induced stomatal closure did not change by 3 and 6 h ozone (300 ppb) exposures (Fig. 3.8b). Among the homologs of ACC synthase genes in Arabidopsis, *AtACS6* is an isogene induced by ozone (Vahala et al., 1998). We thus investigated the ozone-induced expression of this gene in wild-type and the sesquiterpene expressing lines. While the transcript accumulation was 2-3 fold increased in the wild-type and in one of the *tps23* overexpressing lines, L16, the mRNA level was unchanged in the other line (L17) tested (Fig. 3.8c). At ambient air condition, the transcript of this gene was found in significantly larger amount in the transgenic lines compared to the wild-type. We also tested the expression of catalase1 (*CAT1*), an enzyme involved in the breakdown of H_2O_2 after ozone exposure. Interestingly, we observed ozone-induced suppression of this enzyme at both 3 and 6 h exposure in all Arabidopsis genotypes (Fig. 3.8d). Since ozone-induced SA accumulation concomitantly affects the expression of *PR1* gene (Sharma et al., 1996), we analyzed the ozone-induced expression of *PR1* to see if the difference on ozone-induced SA accumulation between the lines also exists for *PR1* expression. With 3 h ozone exposure, the *PR1* transcript was found significantly larger in the wild-type plants (Fig. 3.8e), although the level was 6-fold less compared to the transcript level at 6 h ozone exposure. At 6 h exposure time, the *PR1* transcript was significantly larger in the TPS10 sesquiterpene expressing lines compared to the wild-type or the TPS23 expressing lines.

3.2.6 The TPS10 and TPS23 sesquiterpenes emitted by the transgenic plants degrade ozone in chamber experiments

It is known that ozone reacts rapidly with many biogenic volatile organic compounds; among them are the sesquiterpenes (*E*)-β-caryophyllene and (*E*)-β-farnesene (Kourtchev et al., 2009; Jardine et al., 2011). In order to estimate the ozone quenching abilities of the sesquiterpene-emitting lines, we measured the emission rate of the sesquiterpenes in these plants before, during and after a mild ozone (60 ppb) exposure for 5 to 6 h. We also recorded the corresponding drop in ozone concentration from the preset

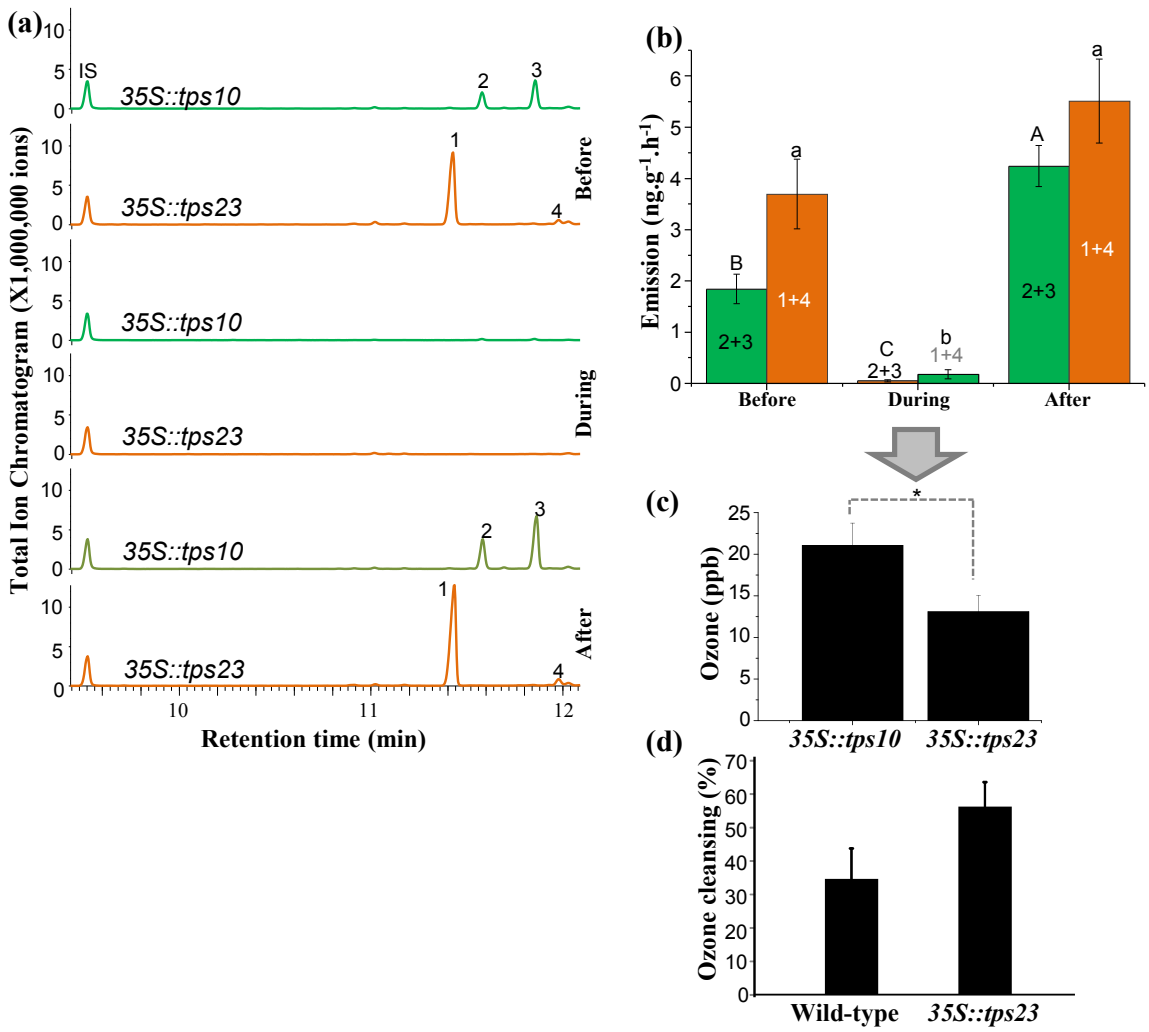

Fig. 3.9: Ozone quenching properties of sesquiterpene overproducing transgenic Arabidopsis plants in fumigation boxes. **(a)** GC-MS chromatogram showing sesquiterpene emission patterns before, during and after ozone exposure with 60 ppb for 5 to 6 h. *(E)*-β-caryophyllene[1] and *α*-humullene[4] are products of the *35S::tps23* construct and *(E)*-α-bergamotene[2] and *(E)*-β-farnesene[3] are products of the *35S::tps*10 construct. **(b)** Quantification of these sesquiterpene products before, during, and after ozone exposure with GC-FID. **(c)** Shows the concentration of ozone detected during exposure in chambers either with the *35S::tps10* (Chamber1) or the *35S::tps23* (Chamber2) plants. **(d)** Shows ozone cleansing capacities of Wt-Col-0 and *35S::tps23* plants tested in a separate experiment.

value in the fumigation chambers. Exposure to such a mild level of ozone nearly abolished the detection of the sesquiterpene volatiles (Fig. 3.9a & b). However, we were able to detect the full emission rates of the plants when we brought them back to the ambient air condition. Interestingly, the emission rates after exposure seemed to be significantly increased compared to the emission rates recorded before exposure, especially for the TPS10 sesquiterpene producing transgenic lines (Fig. 3.9b). Similarly, the chamber with the *35S::tps10* transgenic lines reduced the original ozone concentration on average by 67 percent, while the chamber with the *35S::tps23* lines reduced the

original ozone concentration by 79 percent (Fig. 3.9c). In a separate experiment we have been showing differences in ozone cleansing capacities of wild-type Col-0 and the *35S::tps23* lines (Fig. 3.9d).

As a proof of principle, similar ozone quenching assays were conducted using pure sesquiterpene standards instead of plants. Pure *(E)*-β-caryophyllene representing the *35S::tps23* and *(E)*-β-farnesene representing the *35S::tps10* lines were used. Fumigation chambers flashed with pure n-hexane did not reduce the original ozone concentration (250 ppb) maintained in the chamber (Fig. 3.10). However, when flushed with either 200 ng μl^{-1} *(E)*-β-caryophyllene or *(E)*-β-farnesene, the original ozone concentration in the chambers were dramatically and rapidly reduced by 70 percent and 59 percent respectively.

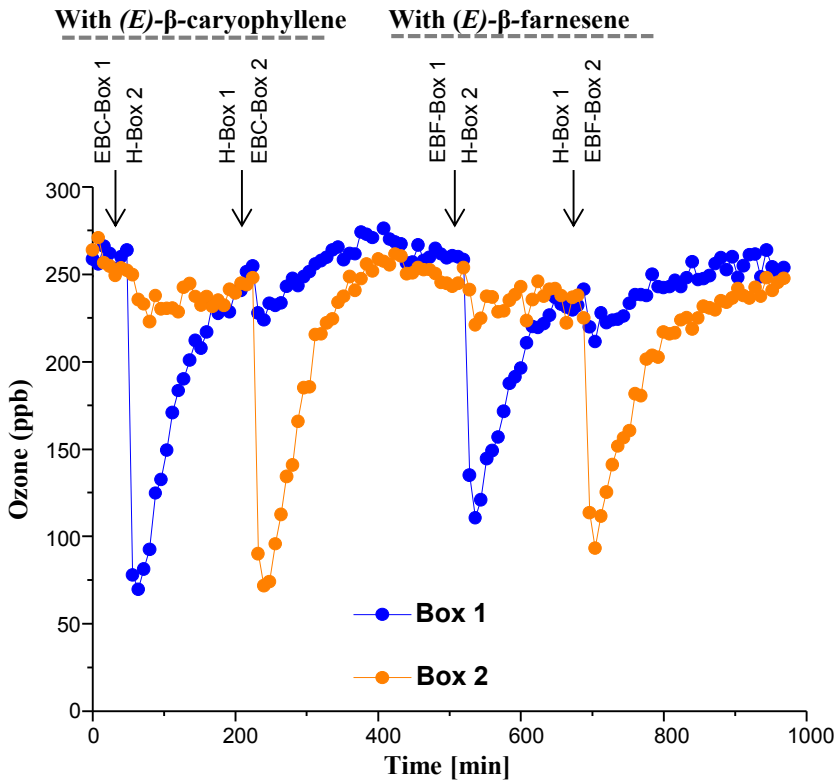

Fig. 3.10: Ozone quenching analysis with pure sesquiterpene standards. Pure *(E)*-β-caryophyllene (EBC) and *(E)*-β-farnesene (EBF) representing the major products of the *35S::tps23* and *35S::tps10* transgenic lines respectively were used in this experiment. Two empty fumigation boxes were initially allowed to stabilize with ozone concentration of 250 ppb. Boxes were then alternately flashed with either pure n-hexane (H) or pure sesquiterpene standards at a concentration of 200 ng dissolved in n-hexene. Arrows above the trend lines show flushing time points. Realtime ozone concentrations were recorded throughout the experimental period. This experiment was repeated three times with similar response patern.

3.3 Discussion

In order to test the hypothesis that sesquiterpenes protect plants against ozone-induced stresses, we generated transgenic Arabidopsis plants that overexpressed two maize sesquiterpene synthase genes, *tps10* and *tps23*, under the control of the CaMV 35S promoter. The genes were preliminarily screened for their high ozone-inducibility in maize (Fig. S9.2.1) and selected for this study since their sesquiterpene products are well known to be highly reactive with ozone (Atkinson and Arey, 2003). In addition, we hypothesized that sesquiterpene emitting plants are better protected from ozone damage due to a significant degradation of ozone in the leaf-atmosphere boundary layer. The ozone is degraded before it enters via stomata and generates a cascade of damaging oxidative products.

To evaluate the responses of transgenic Arabidopsis plants that overproduce *(E)*-β-caryophyllene or *(E)*-β-farnesene-type sesquiterpenes in comparison to wild-type plants at the rosette stage, we analyzed the physiological responses relating to oxidative damage after plants were exposed to an ozone episode of 300 ppb for 6 h. Our results indicated that ozone-induced visible leaf injuries were substantially lower on the sesquiterpene-emitting compared to the wild-type plants (Fig. 3.3), suggesting that the transgenic lines show some degree of resistance against this atmospheric pollutant. This was further reflected by a remarkable reduction in ion-leakage observed in the sesquiterpene-producing lines (Fig. 3.4), an indirect evidence for the relative intactness of membranes in the transgenic lines after ozone exposure.

In order to compare the effect of a short-term ozone exposure on the photosynthetic response and stomatal conductance between sesquiterpene-overproducing and wild-type plants, we conducted gas exchange analysis. We found that the ozone-induced drop in photosynthetic efficiency was faster in the wild-type compared to the sesquiterpene-overexpressing lines. During the first 3 h ozone exposure, a 32 percent reduction in the photosynthetic efficiency was observed in the wild-type plants while the *35S::tps10* and *35S::tps23* lines maintained their ambient level photosynthetic capacity (Fig. 3.5a). Protection of the photosynthetic apparatus from ozone by isoprene was suggested to be achieved through strengthening of the thylakoid membranes (Loreto et al., 2001; Sharkey et al., 2001) and preventing lipid peroxidation (Loreto & Velikova, 2001) under prolonged oxidative stress. A similar kind of function is likely for

sesquiterpenes since both isoprene and sesquiterpenes are similar in that they are olefinic and sufficiently small to dissolve in biological membranes.

Stomatal conductance determines the uptake of ozone into leaf mesophyll (Kollist et al., 2000), and natural variation in ozone sensitivity among Arabidopsis accessions was shown to be governed by differences in stomatal conductance (Brosche et al., 2010). We wanted to exclude the possibility that the sesquiterpene-overproducing plants may somehow accelerate the closure of their stomata to avoid ozone uptake. However, we did not observe any difference on stomatal conductance between the wild-type and the sesquiterpene overexpressing transgenic lines (Fig. 3.5b), indicating that there was not any differential ozone uptake or exclusion caused by stomatal regulation. Since the ozone-induced closure of stomata is controlled mainly by the ABA signaling in concert with the protein phosphatase *ABI1* in the guard cells (Meinhard et al., 2002; Ahlfors et al., 2004; Vahisalu et al., 2010), we analyzed the accumulation of ABA and expression of the *ABI1* gene after ozone exposure. However, we did not find any difference in the ABA accumulation (Fig. 3.7c) and the *ABI1* gene expression (Fig. 3.8b) between the wild-type and the sesquiterpene-overproducing transgenic lines. Therefore, it is unlikely that the sesquiterpenes differentially affect the signaling process to regulate the ozone-induced stomatal closure. It was also reported that differences in ozone sensitivity cannot be always explained by variations in ozone fluxes inside leaves, since ozone resistance in some plant species is also related to their efficient enzymatic and non-enzymatic scavenging mechanism of the ozone itself and the ozone-induced reactive oxygen species (Loreto & Fares, 2007).

Ozone rapidly reacts with cellular components and triggers the accumulation of reactive oxygen species (ROS) which further initiate plant cell death and the development of visible foliar lesions (Rao, et al., 2000). We measured the ozone-induced levels of H_2O_2 and found that the sesquiterpene overproducing plants accumulated far less H_2O_2 than the wild-type plants (Fig. 3.6a), suggesting that the transgenic lines either generate less H_2O_2 or are more efficient in removing it. Production of H_2O_2 beyond a threshold level could cause greater cell death and foliar necrosis in the wild-type plants. Membrane denaturation following the accumulation of H_2O_2 in ozone treated plants results in the accumulation of MDA, an end product of lipid peroxidation (Heath & Parker, 1968). We found that the increased accumulation of H_2O_2 was associated with an increase in the

concentration of MDA in the wild-type plants only (Fig. 3.6b). This indicates that the sesquiterpenes in the transgenic Arabidopsis plants effectively protect membranes against ozone-induced denaturation.

Due to their aerobic life style, plants constantly generate ROSs but are equipped with enzymatic and non-enzymatic mechanisms to remove ROS and maintain their redox homeostasis (Iriti & Faoro, 2008). In order to test whether sesquiterpene expression in Arabidopsis modulates the existing enzymatic mechanism to remove H_2O_2, we investigated the ozone-induced expression of catalase1 (*CAT1*), an enzyme involved in H_2O_2 metabolism. Our result indicated that *CAT1* expression did not differ between the wild-type and sesquiterpene-overproducing lines (Fig. 3.8d), even after ozone exposure. A similar response of *CAT* expression was observed in Arabidopsis previously and it was suggested that ozone regulation of catalase activity might be important at the translational or posttranslational level (Sharma & Davis, 1994). It has been also shown that suppression of isoprene production in hybrid poplar increased the production of antioxidants (Behnke, et al., 2009), suggesting that isoprenoids and other nonvolatile antioxidants function in a compromised way during ozone-induced oxidative stress. Thus, the reduced accumulation of H_2O_2 in the transgenic lines after ozone exposure appears to be a direct interaction of sesquiterpenes with ozone which minimizes ozone entry into the plant system. Also, the sesquiterpenes might scavenge H_2O_2 after ozone degrades to form damaging reactive oxygen species inside the leaf mesophyll. This mechamism has been previously reported in isoprene emitting plants during ozone and high temperature stresses (Loreto & Velikova, 2001; Velikova et al., 2004; Affek & Yakir, 2002).

Since the plant hormones SA, JA, and ethylene play key roles in determining the degree of ozone sensitivity in plants (Kangasjärvi et al., 2005), we compared the ozone-induced accumulations of these phytohormones in the sesquiterpene-overexpressing and wild-type plants. Our results indicated that SA was highly induced after ozone exposure in all plants tested, but the absolute levels were two-fold higher in the wild-type compared to the sesquiterpene-overproducing lines (Fig. 3.7a), suggesting that the sesquiterpenes may increase the resistance against ozone-induced oxidative stress. While a moderate increase in SA concentration initiates antioxidative defense responses, high levels of SA can potentiate the activation of a hypersensitive cell death leading to the apparent ozone sensitivity (Rao et al., 1997; Rao & Davis, 1999). As for SA, optimal JA

levels are required for lesion containment during ozone-induced cell death in plants (Overmeyer et al., 2000). The results showed that JA was slightly induced only in ozone-exposed wild-type Arabidopsis plants, while it was either unchanged or suppressed in some of the transgenic lines (Fig. 3.7b). Although induction of JA is considered a necessary step in the prevention of ozone-induced lesion propagation, it is also possible that JA accumulation is a consequence of more cell death, since the precursor for the JA biosynthesis could originate from membranes from dying cells (Kangasjärvi et al., 2005). Therefore, the reduced JA induction can also be considered as indirect evidence indicating reduced membrane damage in the sesquiterpene-producing lines.

The difference in ozone sensitivity between two Arabidopsis accessions (Col-0 and Ws-2) depends on their difference in ozone-induced ethylene production rates (Tamaoki et al., 2003). However, the sesquiterpene-overproducing lines emitted a similar quantity of ethylene to that detected in the wild-type Col-0 after ozone exposure (Fig. 3.7d). This level of ethylene is probably the threshold level of ethylene required for early step signaling leading to the hypersensitive response. Therefore, the different ozone sensitivity between the wild-type and sesquiterpene-overproducing lines is not dependent on differences in ethylene production. This was further confirmed by the ozone-induced expression profile of the *AtACS6* (Fig. 3.8c) gene, the rate limiting enzyme involved in the biosynthesis of ethylene. Interestingly, the basal expression level of this gene was found significantly higher in one of the transgenic lines, although we could not detect any differences in ethylene emissions.

To determine whether the wild-type and sesquiterpene-overexpressing plants differ in their activation of ozone-induced defense genes, we analyzed the expression of *PR1* which is known to be regulated by ozone-induced SA accumulation in Arabidopsis (Sharma et al., 1996). We found out that the expression of *PR1* was faster in wild-type plants compared to the transgenic lines during the beginning of the ozone time course, but at the last time point transcript level was not significantly different or less than that in transgenic lines (Fig. 3.8e). Although activation of SA-dependent *PR1* expression may provide protection from ozone stress (Sharma et al., 1996), the faster induction of *PR1* may be due to a quicker and more profound damage by ozone and a sharper rise in SA concentrations (Fig. 3.7a) in wild-type than in the sesquiterpene-overproducing transgenic lines.

It has been suggested that isoprene can directly react with ozone either *in planta* or at the leaf surface to decrease the level of ozone and so reduce toxicity to plants (Loreto et al., 2001a&b; Vickers et al., 2009). Our findings indicated that a similar ozone quenching property can be observed with the transgenic Arabidopsis lines overproducing *(E)*-β-caryophyllene or (*E)*-β-farnesene-type of sesquiterpenes, where a 20 to 35 percent drop in the ozone concentration was seen (Fig. 3.9). We confirmed that the gas phase reaction of ozone with the sesquiterpenes may contribute to the significant destruction of ozone in the leaf boundary of the transgenic plants. This was shown by a quenching experiment using realistic concentrations of authentic *(E)*-β-caryophyllene or (*E)*-β-farnesene in chamber experiments (Fig. 3.10). The results suggest that the emission of sesquiterpenes acts as an effective ozone sink around the leaf boundary and significantly reduces ozone uptake and the associated oxidative damage. A similar mechanism of protection has been proposed for gas-phase reactions with monoterpenes (Pinto et al., 2007a; Fares et al., 2008) and sesquiterpenes (Jardine et al. 2011). But, one cannot rule out the possibility that these sesquiterpenes scavenge ozone-induced toxic reactive oxygen species *in planta*, as has been shown for isoprene in Rose Bengal treated plants (Affek & Yakir, 2002; Velikova et al., 2004). Due to its lipophilic properties, isoprene was proposed to stabilize membrane under heat stress (Sharkey & Singsaas, 1995). This assumption was lately verified with molecular dynamic simulation techniques showing isoprene protecting a model phospholipid membrane from heat-induced phase transitions (Siwko et al., 2007). Given that sesquiterpenes also tend to be lipophilic, a similar membrane stabilization and protection from ozone-induced membrane damage could be achieved by sesquiterpenes. Whether sesquiterpenes scavenge reactive oxygen species in intracellular space or play a role in membrane stability, however, could not be determined with the current experiments.

3.4 Materials and Methods

3.4.1 Transformation of Arabidopsis plants with the maize tps10 *and* tps23 *genes*

The open reading frame of the maize B73 *tps10* and Delprim *tps23* genes were amplified by PCR from the pASK-IBA7 (Schnee et al., 2006) and pHIS8-3 (see Köllner et al. 2008) vector constructs respectively with gene specific primer sets (Table S9.2.1).

The amplified sequences were independently cloned in to the pCR-TOPO vector (Invitrogen, Carlsbad, CA) for sequencing. After sequence analysis, the 1,602 bp of *tps10* and 1,644 bp of *tps23* were re-amplified from the sequencing vector and independently cloned into the plant expression vector pB2GW7 using Gateway cloning technology between the *35S* promoter and the *T35S* terminator of the cauliflower mosaic virus. The resulting constructs were introduced into *Agrobacterium tumefaciens* strain GV3101, which was then used to transform two *Arabidopsis thaliana* ecotypes (Col-0 and Ws-2). Since the constructs have a *bar* selectable marker gene that confers resistance to the herbicide Basta (Glufosinate), T_1 plants were screened for the transgene by applying Basta on soil germinated young seedlings. To select transgenic lines emitting the expected TPS10 and TPS23 sesquiterpenes, headspace volatiles were collected from detached leaves of the Basta survivors with SPME (solid phase micro extraction) and analyzed by GC-MS. Two independent T_2 lines from each gene transformation were selected for further ozone fumigation experiments.

3.4.2 Plant volatile collection and analysis

A dynamic headspace volatile collection system, which was installed in a climate controlled chamber (20/18 ^{o}C day/night temperature, 50 percent relative humidity, 150 µmol $m^{-2}s^{-1}$ photosynthetically active radiation, 8/16 hrs light/dark condition) was employed to collect volatiles from wild-type and transgenic Arabidopsis plants. In brief, five-week old Arabidopsis plants were placed independently in a 3 l glass cylinder (Schott; Jena, Germany) which has air inlet and outlet valves on the lid of the cylinder. Charcoal filtered air was allowed to enter to the cylinder with adjusted flow rate of 2 l min^{-l} through Teflon tubing. After mixing with the volatiles emitted from the plant, the air exited the cylinder through a volatile collection trap, 150 X 6.4 mm glass tube (Analytical Research Systems, Gainesville, FL, USA) containing 30 mg Super Q material (Alltech, Deerfield, IL) which was fixed inside the air outlet valve. All volatile collections were conducted during the light phase in a similar time frame (8:00 am-2:00 pm) for a period of 6 consecutive hours. After the collection, the volatiles trapped were eluted with 200 µl dichloromethane containing 5 ng μl^{-1} nonyl acetate as an internal standard and analyzed by GC-MS. A Hewlett-Packard model 6890 gas chromatograph was used with carrier gas He at 1 ml min^{-1}, splitless injection (injector temperature, 220 ^{o}C; injection volume, 2 µl), a DB-5MS column (30 m x 0.25 mm x 0.25 µm film; J and W Scientific, Folsom, CA,

USA), and a temperature program from 40 ^{o}C (2 min hold) at 5 ^{o}C min-1 to 240 ^{o}C (2 min hold). The coupled mass spectrometer was a Hewlett-Packard model 5973 with a quadrupole mass selective detector, transfer line temperature of 230 ^{o}C, ionization potential of 70 eV, and a scan range of 50 to 400 m/z. Compounds were identified by reference spectra in the Wiley and National Institute of Standards and Technology libraries and in the literature (Schnee et al., 2006; Köllner et al., 2008). For accurate quantification, compounds were analyzed by GC with a flame ionization detector (FID) operated at 250 ^{o}C and using the carrier gas H_2 at 2 ml min^{-1}. Peak areas were compared with that of the internal standard by applying a response factor of 1 for the internal standard and 0.74 for all the sesquiterpenes identified, as calculated according to the effective carbon number concept (Scanion & Willis, 1985).

3.4.3 Ozone fumigation system

Ozone was generated from oxygen by electrical discharge in a SORBIOS ozone generator (Berlin, Germany). Ozone fumigation was performed in boxes made of Plexiglas (0.5 m^3) with continuous gas stirring inside. The boxes were connected to Teflon tubing to introduce ozone and ambient air to the fumigation system and to take air samples for ozone and volatile analysis from the system. Ozone concentrations in the boxes were continuously monitored through a UV photometric ozone analyzer (Ansyco, Karlsruhe, Germany). Charcoal-filtered air was pumped into the experimental chambers with adjusted flow rates that generate the required ozone concentrations in the fumigation boxes. The boxes were located in a dedicated controlled climate chamber which was adjustable for either maize (24/20 ^{o}C day/night, 60 percent RH, with 14 h photoperiod at 500 µmol m^{-2} s^{-1}) or Arabidopsis (20/18 ^{o}C day/night, 50 percent RH, with 8 h photoperiod at 150 µmol m^{-2} s^{-1}) fumigation experiments. Acute exposure to ozone consisted of a single 6 h fumigation, between 8:00 am-2:00 pm, at a concentration of 300 ppb unless mentioned otherwise. The whole setup used in this experiment is shown in Figure 3.2.

3.4.4 Histochemical staining

Accumulation and localization of hydrogen H_2O_2 in leaves of Arabidopsis and maize after ozone treatment was determined following the protocol described in (Thordal-Christensen et al., 1997). In brief, leaves were excised at the base of the petioles in the

case of Arabidopsis and at the base of the leaf sheath in the case of maize and immersed in a 1 mg/ml solution of 3,3´-diaminobenzidine (DAB), pH 3.8 for 6 h in the controlled climate chambers. In the presence of H_2O_2, DAB polymerizes turning to a reddish-brown color visually assessed by scanning (HP Scanjet 5590P) at a resolution of 1200 dpi after terminating the experiment by boiling samples in 96 percent ethanol for 10 min.

Cell death after ozone fumigation in leaves of Arabidopsis and maize was visualized by boiling leaves in lactophenol (lactic acid:glycerol:liquid phenol:distH_2O in 1:1:1:1 ratio) containing the same proportion of trypan blue for 1 min (Rate et al., 1999). Immediately after boiling, tissues were cleared in alcoholic lactophenol (95 percent ethanol:lactophenol in 2:1 ratio) for 2 min and washed in 50 percent ethanol at room temperature. Tissues were visualized by scanning (HP Scanjet 5590P) at a resolution of 1200 dpi. Additionally, ozone-induced visible damage was recorded and compared on whole rosette leaves of wild-type and sesquiterpene expressing transgenic Arabidopsis plants 3 days after ozone fumigation by photography with a digital camera.

3.4.5 Ion leakage measurement

Ozone-induced membrane damage was indirectly quantified by measuring the relative ion leakage as described in (Tamaoki et al., 2003). Leaf-disks of 14 mm diameter were collected 6 h after the onset of ozone exposure and submerged in 10 mL Milli-Q water in 50 mL Falcon tubes. The electrical conductivity ($\mu S\ cm^{-1}$) of the water bath was measured with a conductivity meter (WTW GmbH, Weilheim, Germany) after 3 h of incubation at room temperature (EC_1). Total electrolyte content of the leaf disks were estimated by measuring the conductivity of the same samples after incubating in a 95 ^oC water bath for 20 min (EC_2). The relative tissue damage or intactness of membrane was determined by calculating the percent ion-leakage of the leaf disks (EC_1/EC_2 X 100).

3.4.6 Determination of H2O2 content

In vivo levels of H_2O_2 in leaves were measured according to Velikova et al. (2000) with a slight modification. Briefly, samples were collected in liquid nitrogen and ground into fine powder in a pre-cooled mortar and pestle. To a 100 mg aliquot of ground tissue, 1 mL of 5 percent (w/v) trichloroacetic acid (TCA) was added and briefly mixed by vortexing. The homogenate was centrifuged at 10,000*g* at 4 ^oC for 20 min and 0.5 mL of

the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH=7.0) and 1 mL of 1 M KI. The absorbance of the assay mixture was measured at 390 nm in a 96-well plate format (Greiner bio-one, Frickenhausen, Germany) with a photometer (Tecan Infinite, Männedorf, Switzerland). The concentration of H_2O_2 was calculated by comparing the responses with a standard calibration curve made in the same plate using known concentrations of authentic H_2O_2.

3.4.7 Determination of MDA content

As an index of lipid peroxidation, MDA content was measured using the thiobarbituric (TBA) test, which determines MDA as an end product of lipid peroxidation (Heath & Parker, 1968). In brief, samples were collected in liquid nitrogen and homogenized into a fine powder in a pre-cooled mortar and pestle. To a 100 mg aliquot of ground tissue, 1 mL of 5 percent (w/v) trichloroacetic acid (TCA) was added and briefly mixed by vortexing. The homogenate was centrifuged at 10,000*g* at 4 ^{o}C for 20 min and 0.5 mL of the supernatant was mixed with 1 mL of 0.5 percent (w/v) TBA in 20 percent TCA. The reaction mixture was incubated in a water bath at 95 ^{o}C for 30 min and the reaction terminated by placing the tubes in an ice bath. The samples were then centrifuged again at 10,000*g* and 4 ^{o}C for 5 min. The absorbance of the supernatant was measured at 532 nm in a 96-well plate format (Greiner bio-one, Frickenhausen, Germany) with a photometer (Tecan Infinite, Männedorf, Switzerland) and the value of non-specific absorption at 600 nm was subtracted. The amount of MDA was estimated by comparing with a standard calibration curve made in the same plate using known concentrations of MDA.

3.4.8 Gas exchange measurement

Gas exchange measurements were conducted using a portable infrared gas analyzer, LI-6400 XT (LI-COR Environmental, Lincoln, Nebraska, USA), which was coupled with a 6 cm^{-2} exposure cuvette. For whole rosette Arabidopsis gas exchange measurements, a custom made, 7 cm diameter external measuring chamber was connected to the core system. All measurements were conducted at a CO_2 mixing ratio of 350 µmol mol^{-1}, 150-350 µmol $m^{-2}s^{-1}$ photosynthetically active radiation (PAR), RH of 30-40 percent, airflow of 0.1 L min^{-1}, and leaf temperature of 20-24 ^{o}C. The saturated CO_2 assimilation rate (A_{sat}) and stomatal conductance (g_{st}) was recorded after leaves or

rosettes were allowed to adapt in the measuring chamber or cuvettes for 10 min to attain steady-state CO_2 and H_2O fluxes and the results were corrected to the total leaf area measured. All gas exchange measurements were conducted immediately after the end of the respective ozone treatments.

3.4.9 Phytohormone extraction and analysis

For SA, JA and ABA determinations, plants were first fumigated with acute ozone (300 ppb, 6 h) and allowed to recover from the treatment for 3 h. Samples were collected, flash frozen in liquid nitrogen, and stored at minus 80 ^{o}C until use. Samples were ground into a fine powder with mortar and pestle in liquid nitrogen. Approximately 200 mg of frozen ground tissue was transferred to 2 ml Eppendorf tube containing 500 mg FastPrep matrix (BIO 101, Vista, USA) and 0.5 mL extraction buffer (MeOH) spiked with 10 ng D_2-JA, D_4-SA, and D_6-ABA as internal standards was added and homogenized in a reciprocal shaker for 5 min. Samples were vigorously vortexed for 5 min and centrifuged at 8,000*g* for 20 min at 4 ^{o}C. Then, 300 µl of the supernatant was collected in an HPLC vial for the analysis. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies, Boeblingen, Germany).

Ethylene emission was measured with a photo-acoustic spectrometer (INVIVO, Sankt Augustin, Germany) as described by von Dahl et al. (2007). In brief, six-week old Arabidopsis whole rosettes or the third leaf of 15 day old maize seedlings were taken for ethylene collection in a 250 mL flask immediately after the plants were fumigated with 300 ppb ozone for 6 h. After five hours of continuous ethylene collection, the flasks were flushed with a flow of purified air at 128 ml min^{-1} into the sampling cell. To remove hydrocarbons, air was cleaned by oxidizing all organic compounds via passage through a platinum catalyst at 450 ^{o}C (SylaTech, Walzbachtal, Germany) before being directed to the sampling unit. A CO_2 laser for chopping the gas and a resonant photo-acoustic device for the detection were used. The spectrum of ethylene in the infrared region allows for a highly sensitive analysis by altering measurements of the photo-acoustic signal on the CO_2 laser lines 10p14 and 10p16. The detection device consisted of two acoustic cells. One cell was filled with a known ethylene concentration (516 ppb) from a calibration gas reservoir, which was used to calibrate and continuously adjust the laser line. The sampling cell was calibrated with the gas (516 ppb) before the start of each experiment.

3.4.10 Extraction of RNA, cDNA synthesis, and qRT-PCR

To investigate changes in the expression of *pathogenesis-related protein1* (*PR1*), *isochorismate synthase1* (*ICS1*), *mitogen-activated protein kinase3* (*MPK3*), and *abscisic acid insensitive1* (*ABI1)* genes in response to ozone exposure in Arabidopsis, qRT-PCR experiments were conducted on ozone-exposed Arabidopsis leaf samples. In brief, total RNA was extracted from 100 mg fresh Arabidopsis tissue using the Qiagen RNeasy Plant Mini kit (Hilden, Germany) according to the manufacturer's instructions. To remove residual genomic DNA, the extracts were treated with RNAse-free DNAse (Qiagen, Hilden, Germany). RNA quality was measured with an Agilent 2100 Bioanalyzer (Waldbronn, Germany) and quantified spectrophotometrically with a Nanodrop 2000C (Thermo Fisher Scientific, Wilmington, DE, USA). For cDNA synthesis, Superscript III reverse polymerase (Invitrogen, Carlsbad, USA) was used according to the manufacturer's instructions but with 5 µg total RNA in a 20 µl reaction volume. The qRT-PCR was performed on 1:50 diluted cDNA templates in a Stratagene Mx3000P Real-time PCR machine (La Jolla, CA, USA) using SYBR Green-based (Applied Biosystems, Darmstadt, Germany) detection of dsDNA synthesis. The products of each primer pair were cloned and sequenced to verify primer specificity. The linear range of template concentration to threshold cycle value (C_t) was determined by performing a series of six-fold dilutions using cDNA from three independent RNA extractions. All primers were designed in the 3´ UTR of each target gene using Beacon primer design software (Primer Biosoft, Palo Alto, USA) and ordered with HPLC-purification (Invitogen). Primer efficiencies were calculated using the standard curve method (Pfaffl, 2001). The relative transcript level was calculated using actin as the normalizer gene and wild-type Col-0 as calibrator. A similar expression analysis was conducted on the maize B73 *tps10* and Delprim *tps23* genes in response to a single 6 h ozone (300 ppb) exposure. All primer pairs used for the qRT-PCR are listed in Table S9.2.1.

3.4.11 Ozone quenching determination

The ozone quenching capacities of the TPS10 and TPS23 sesquiterpenes were determined by using transgenic plants producing the respective sesquiterpenes. In brief, groups of 15 *35S::tps10* or *35S::tps23* transgenic plants were inserted in two fumigation boxes separately. Then, the emission rate of the respective sesquiterpenes was measured in these plants before, during and after a mild ozone (60 ppb) exposure for 6 h (8:00 am-

2:00 pm). At the same time, the corresponding drop in the ozone concentration from the originally set value was recorded during the ozone exposure time. A similar quenching experiment was conducted by directly injecting 200 ng μl^{-1} authentic sequiterpene standards, *(E)*-β-caryophyllene or *(E)*-β-farnesene, into the fumigation chambers to mimic the sesquiterpene emitting transgenic plants. The same volume of pure *n*-hexane was flushed into the fumigation boxes as a control to mimic the non-sesquiterpene emitting wild-type plants. The drop in the ozone concentration was recorded continuously online until the two boxes read similar ozone concentrations. The percent ozone degraded in each box was calculated and compared to the corresponding decrease in the emission rate of the sesquiterpenes.

4. Research Chapter III

Restoring *(E)*-β-caryophyllene production in a non-producing maize line compromises its resistance against the fungus *Colletotrichum graminicola**

Abstract

The sesquiterpene *(E)*-β-caryophyllene is emitted from maize (*Zea mays*) leaves and roots in response to herbivore attack. This compound serves as a signal for the attraction of herbivore enemies, including entomopathogenic nematodes and parasitic wasps, and is present in most European maize varieties. However, most North American maize lines have lost the ability to produce *(E)*-β-caryophyllene. Previously it has been shown that genetically restoring the ability to synthesize *(E)*-β-caryophyllene in a non-producing maize line improved its resistance against the root herbivore *Diabrotica virgifera virgifera*. However, it is largely unknown whether this restoration affects the resistance of the plant to other pests. In this study we investigated the response of the constitutively *(E)*-β-caryophyllene-producing transgenic lines to infection by the fungus *Colletotrichum graminicola.* Our results showed that restoring *(E)*-β-caryophyllene synthesis in a Hi-II genetic background enhanced the susceptibility of the plant to *C. graminicola* infection rather than increasing its resistance. This modification did not alter the baseline levels of plant defense hormones or benzoxazinoids, a group of anti-fungal and anti-herbivore defense metabolites. Nor did *(E)*-β-caryophyllene production modify the expression of anti-fungal defense genes after infection. Instead, the addition of *(E)*-β-caryophyllene seemed to directly stimulate fungal growth. In an *in vitro* antifungal assay, we found that *(E)*-β-caryophyllene stimulated hyphal growth of *C. graminicola* and *Fusarium graminearum.* Thus, although restoring *(E)*-β-caryophyllene production in a non-producing maize line may improve the resistance of the plant against herbivores, it may compromise its resistance to major fungal pathogens. This compound appears to be employed as a host-finding cue by *C. graminicola* and *F. graminearum* for successful colonization of the plant and this may explain its loss during maize breeding in environments where such pathogens are prevalent.

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4.1 Introduction

Plants produce a wide spectrum of volatile organic compounds that may mediate mutualistic or antagonistic interactions with other organisms. For instance, volatiles function as cues for the attraction of pollinators (Knudsen et al., 2006) or as toxins for defenses against herbivores (Bernasconi et al, 1998; De Moraes et al., 2001; Junker et al, 2011). Additionally, these volatiles can be employed as signals to attract natural enemies of herbivores (Dicke, 2009) or for plant-to-plant communication (Baldwin et al., 2006). Although plant volatiles consist of many different structural types, the majority of these metabolites are terpenoids, comprising over 40,000 different structures (Tholl et al., 2004; Aharoni et al., 2005).

We have been using maize as a model to study the biochemical and molecular aspects of terpene formation. Maize can synthesize and emit nearly 100 different terpenoid volatiles dominated by sesquiterpene hydrocarbons (Köllner et al., 2004a&b). Most of these compounds are formed by several multiproduct terpene synthases which are expressed differentially throughout the plant (Köllner et al., 2004a; Schnee et al., 2006; Köllner et al., 2008). The herbivore-induced terpene synthase TPS10 produces a sesquiterpene blend dominated by *(E)*-β-farnesene and *(E)*-α-bergamotene in leaves (Schnee et al., 2006). At a later developmental stage, maize emits volatile mixtures from its mature leaves and husks dominated by olefinic sesquithujene- and bisabolene-type sesquiterpenes. These volatiles are produced by the TPS4 or TPS5 sesquiterpene synthases (Köllner et al., 2004a). The terpene synthases TPS7 and TPS8 are responsible for the production of the constitutively emitted sesquiterpenes dominated by germacrene D and δ-cadinene (Fontana, PhD dissertation). Last but not least, the sesquiterpene *(E)*-β-caryophyllene is emitted by maize leaves and roots after herbivory. The enzyme responsible for the biosynthesis of this compound was characterized as the sesquiterpene synthase 23 (TPS23; Köllner et al., 2008).

In the last decade, a series of studies have been conducted to unravel the ecological relevance of these volatile sesquiterpenes as defense compounds against herbivores. Several of these studies used transgenic *Arabidopsis thaliana* plants engineered with maize sesquiterpene synthase genes. For instance, to test the function of the TPS5 sesquiterpene volatiles in indirect defense, transgenic *A. thaliana* lines over-expressing the maize TPS5 enzyme were employed in an olfactometer experiment to

demonstrate that the TPS5 volatiles attract parasitoids of lepidopteran larvae (Fontana et al., 2011). Similarly, transgenic Arabidopsis plants emitting the TPS10 sesquiterpenes attracted the parasitoid *Cotesia marginiventries* (Schnee et al., 2006). The function of the sole TPS23 product, *(E)*-β-caryophyllene, was tested by restoring *(E)*-β-caryophyllene production to a maize line where formation of this sesquiterpene had been lost in breeding (Degenhardt et al., 2009). After *(E)*-β-caryophyllene emission, a property of the ancestor of cultivated maize, teosinte, was restored by genetic transformation with the *(E)*-β-caryophyllene synthase gene from oregano, the transformants were found to attract entomopathogenic nematodes below ground which attacked larvae of the coleopteran *Diabrotica virgifera virgifera* and reduced root damage (Degenhardt et al., 2009). This compound was also demonstrated to be a crucial signal in attracting lepidopteran parasitoids to damaged shoots above ground (Köllner et al., 2008).

Maize sesquiterpenes have also been demonstrated to act as defenses against pathogens. The non-volatile sesquiterpenes zealexins, which are derived from a biosynthetic pathway involving TPS6 and TPS11 in maize, have been shown to serve as inducible anti-fungal defenses (Huffaker et al., 2011). Volatile sesquiterpenes may also be involved in defensive roles vs. pathogens, but much less research has been carried out on this topic using genetically manipulated plants as compared to the work on anti-herbivore defenses. For example, studies manipulating the terpene synthase genes of *Arabidopsis thaliana* revealed that *(E)*-β-caryophyllene, the major sesquiterpene volatile released from flowers, reduced infection by a bacterial pathogen (Huang et al., 2012). In addition, there is a vast literature on terpenoid-containing essential oils indicating that these compounds have antifungal activities (Cavanagh et al., 2007). However, a few recent studies demonstrated the opposite effect. For instance, antisense down-regulation of terpene production in sweet orange plants unexpectedly improved the resistance of the plants to economically important fungal pathogens (Rodriguez et al., 2011). In an *in vitro* anti-fungal assay, several citrus terpenes were reported to have pronounced stimulatory effects on germination and germ tube elongation of fungal pathogens (Droby et al., 2008). Although many of the major terpene volatiles in these plants are monoterpenes, sesquiterpenes like *(E)*-β-caryophyllene are present in the oils as well. Interestingly, citrus cultivars that emit *(E)*-β-caryophyllene as part of their volatile bouquet support the highest germination percentage of fungal pathogens (Droby et al., 2008). Some fungus species also induce the production of volatiles in their hosts and subsequently employ

these volatiles as cues for the stimulation of growth. For instance, the rust fungus *Uromyces fabae* has been shown to stimulate the volatile emission of its host *Vicia faba* (Mendgen et al., 2006). In an *in vitro* anti-fungal assay, some of the induced volatiles have been shown to be perceived by the fungus for the promotion of haustorial growth.

One conspicuous feature of plant volatile terpenes is the variation in composition among different varieties or ecotypes within a species (Köllner et al., 2004a&b; Tholl et al., 2005). For example, in maize the genetic variability of induced volatiles among inbred lines commonly used by North Ameriacn and European breeders was described by Degen et al (2004). European and North American lines typically differ in their release of the sesquiterpene *(E)*-β-caryophyllene, with emission being much more common among the European lines as in the maize ancestor teosinte, and rarer in the North American lines. *(E)*-β-caryophyllene emission in turn leads to increased resistance to maize root herbivores by acting as an attractant for herbivore enemies (Rasmann, et al., 2005; Degenhardt et al., 2009). Genetic variability based on *(E)*-β-caryophyllene production has been also demonstrated in two other maize inbred lines, Mp708 and Tx601, where a significant difference in resistance against a lepidopteran larva was observed (Shivaji et al., 2010; Smith et al., 2012). Information on the impact of this variability on the resistance of maize lines against microbial pathogens is more limited. However, a recent study by Erb et al (2011) demonstrated that most dent maize genotypes (*Z. mays* indentata) commonly grown in North America are more resistance to the fungus *C. graminicola* than the flint genotypes (*Z. mays* indurate) which are commonly grow in Europe and are able to produce *(E)*-β-caryophyllene.

In this study, we investigated the effect of restoring the production of the sesquiterpene *(E)*-β-caryophyllene in a non-producing maize line Hi-II on its resistance to the pathogenic fungus *C. graminicola*, a common fungal pathogen of maize (*Zea mays*) that seriously reduces grain yield and quality by causing stalk rot and anthracnose in most maize tissues (Bergstrom & Nicholson, 1999; Sukno et la., 2008). The effect of *(E)*-β-caryophyllene was also studied with *Fusarium graminearum*, another maize pathogen that causes maize ear and stalk rot, and seedling blight (Presello et al., 2006; Vigier et al.., 2001). The results indicate that *(E)*-β-caryophyllene increased the susceptibility of maize to fungal infection possibly by stimulating fungal growth. The presence of *(E)*-β-caryophyllene did not affect the levels of various defense hormones or antifungal defense compounds in the plant or the expression of other defense genes investigated.

4.2 Results

4.2.1 Restoring *(E)*-β-caryophyllene production in non-producing maize line

The maize inbred line Hi-II that had lost the ability to produce *(E)*-β-caryophyllene found in the ancestor to cultivated maize was transformed with the *Origanum vulgare (E)*-β-caryophyllene synthase gene (*tps6*) placed under the control of a maize ubiquitin promoter (Degenhardt et al., 2009). The transgenic Hi-II lines were able to constitutively produce and emit *(E)*-β-caryophyllene as a major product and α-humulene as a minor product, whereas these two sesquiterpenes were not detected in the headspace as well as tissue pentane extracts of wild-type plants (Fig. 4.1a). The transgenic plants were able to produce these sesquiterpenes both in leaves and roots.

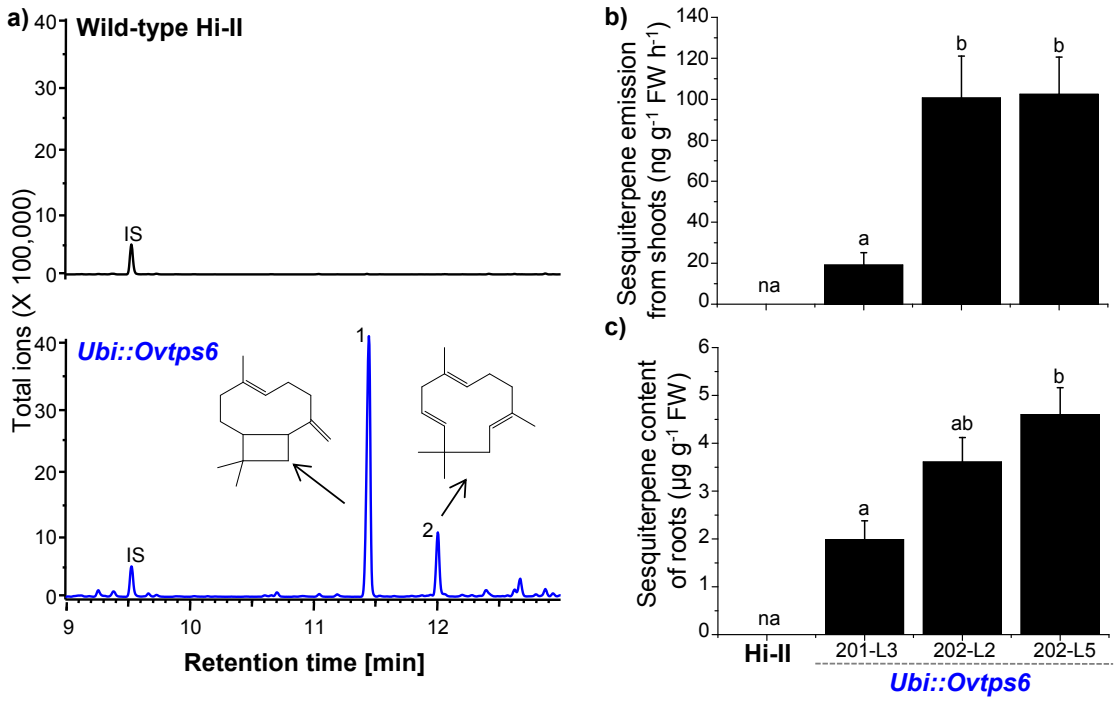

Fig. 4.1: *(E)*-β-caryophyllene production in transgenic Hi-II maize line overexpressing the *Origanum vulgare* L. caryophyllene synthase gene (*tps6*). (**a**) Headspace volatile collection from young seedlings of wild-type (Hi-II) and transgenic (*ubi::Ovtps6*) maize lines analyzed via GC-MS. The sesquiterpenes identified in the transgenic lines are *(E)*-β-caryophyllene[1] and *α*-humulene[2]. Nonylacetate was used as an internal standard (IS). (**b**) The shoot emission rate of the two sesquiterpenes combined as quantified by GC-FID. Bars represent mean ± SE of n = 12 replicates. (**c**) The combined sesquiterpene concentration extracted from the roots by pentane and quantified by GC-FID. Bars represent mean ± SE of n = 6-12 replicates. Bars with different letters represent significant differences after ANOVA followed by Tukey HSD post hoc test at α = 0.05.

Three independent selfed T_2 transgenic lines were selected for further experiments. The emission rate of the sesquiterpenes in two of the transgenic lines (202-L2 and 202-L5) was almost similar (100 ng h^{-1} g^{-1} fresh weight, Fig. 4.1b); whereas in the third transgenic line (201-L3), the emission rate was five-fold less (20 ng h^{-1} g^{-1} fresh weight) compared to the other two transgenic lines. We also detected these two sesquiterpenes in the roots in the range of two to five ng g^{-1} fresh weight using tissue pentane extraction (Fig. 4.1c) and this level was proportional to the leaf emission rate observed.

4.2.2 Restoring *(E)*-β-caryophyllene production in non-producing Hi-II lines increased susceptibility to *C. graminicola* infection

To test whether restoring *(E)*-β-caryophyllene production in non-producing Hi-II maize lines confers resistance or susceptibility to the causal agent of anthracnose disease, seedlings were inoculated with *C. graminicola* spores and assessed for disease symptoms and fungal development. Infected plants did not show remarkable visible disease symptoms during the first four days post inoculation. However, seven days after inoculation, the two transgenic lines (202-L2 and 202-L5) that had high *(E)*-β-caryophyllene emission rates developed greater symptoms compared to the wild-type and the transgenic line with the low emission rate (201-L3) (Fig. 4.2a). The disease score based on a scale from 0 to 10 revealed that the transgenic lines with higher emission rates showed a significantly greater disease score compared to the wild-type Hi-II (Fig. 4.2b). When we assessed the growth of the fungus in the plant tissue by quantifying a fungal structural gene using PCR, significantly larger accumulation of *C. graminicola actin* was detected in at least one of the high *(E)*-β-caryophyllene producing transgenic lines (Fig. 4.2c).

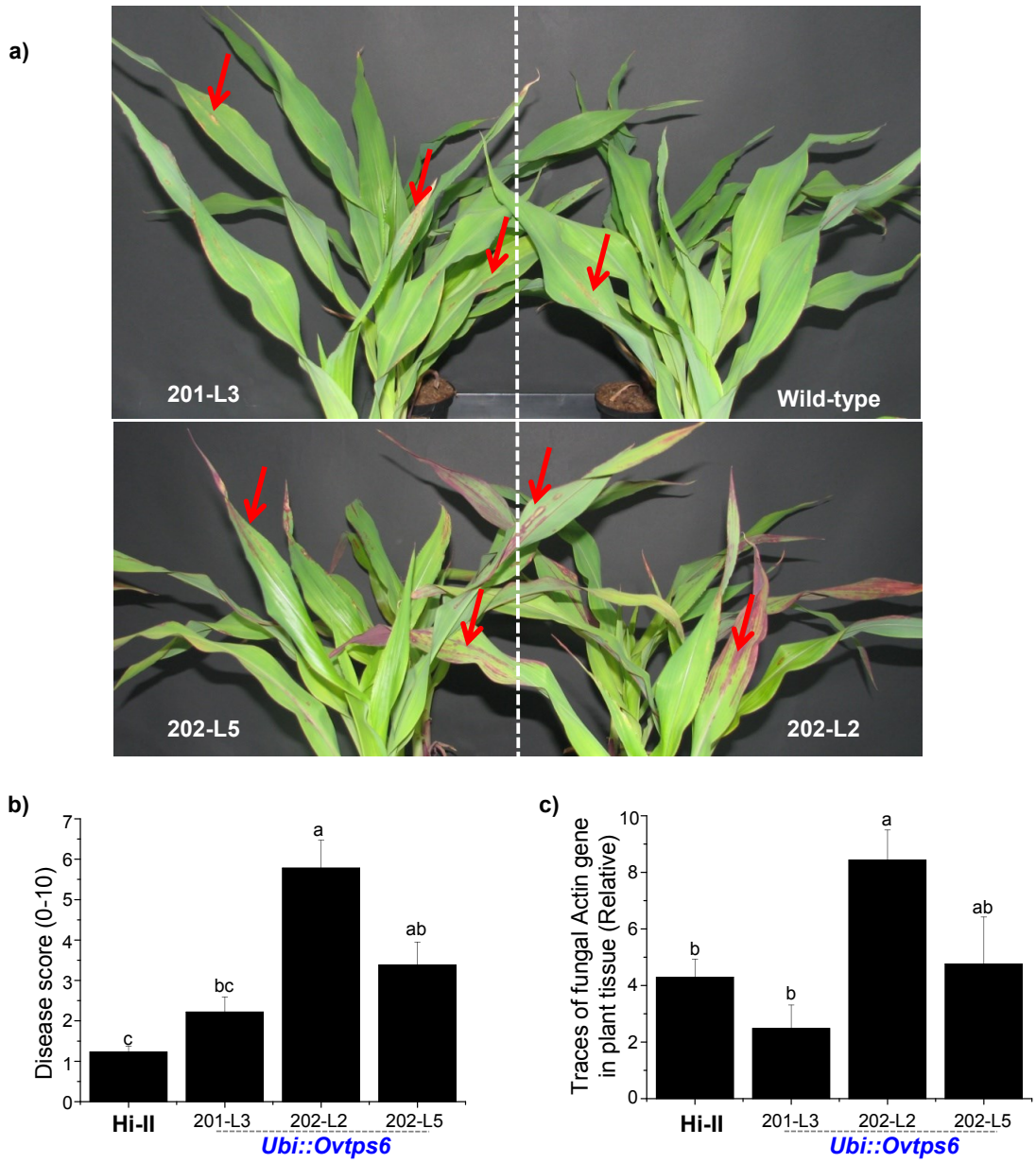

Fig. 4.2: Anthracnose disease assesment in *C. graminicola* infected wild-type (Hi-II) and E-β-caryophyllene producing transgenic Hi-II maize lines (201-L3, 202-L2, and 202-L5). (**a**) Photographs showing anthracnose disease symptoms 7 days post inoculation (7 dpi). (**b**) Disease score calculated based on leaf area displaying disease symptom; "0" was assigned for no symptom and "10" for fully infected laves. (**c**) Relative quantity of fungal biomass in infected plant tissues as quantified by PCR based on amplification of *C. graminicola actin* gene from gDNA isolated from infected plants. Bars with different letters show significant differences at α = 0.05 after ANOVA followed by Tukey HSD analysis.

4.2.3 *(E)*-β-caryophyllene stimulates the growth of *C. graminicola* and *F. graminearum in vitro*

To determine the growth inhibitory or stimulatory effect of *(E)*-β-caryophyllene on two major maize pathogenic fungi, *C. graminicola* and *F. graminearum*, an *in vitro*

fungal growth assay was conducted on PDA (Potato Dextrose Agar) plates containing different concentrations of (*E*)-β-caryophyllene. Concentration levels of 50 and 100 ng μl^{-1} PDA solution stimulated both fungal species to grow faster compared to plates with no (*E*)-β-caryophyllene (Table 4.1). While the 200 ng μl^{-1} concentration did not cause any stimulatory or inhibitory effect on *C. graminicola* growth, the 500 and 1000 ng μl^{-1} concentrations showed a remarkable growth inhibitory effect. In *F. graminearum*, only the 1000 ng μl^{-1} *(E)*-β-caryophyllene concentration had a clear inhibitory effect on the fungal growth. At day 4, the 500 ng μl^{-1} *(E)*-β-caryophyllene concentration also showed inhibitory effect. The 50 and 100 ng μl^{-1} *(E)*-β-caryophyllene concentrations showed significant growth stimulatory effects but only at later measurement times (day 3 and 4).

Table 4.1: Mean (±SE) growth response of *C. graminicola* and *F. graminearum* on *(E)*-β-caryophyllene (EBC) amended potato dextrose agar plates

EBC (ng/µl)	*C. graminicola* colony radius (mm)					*F. graminearum* colony radius (mm)				
	Day1	Day2	Day3	Day4	Day5	Day1	Day2	Day3	Day4	Day10
0	6.42 ± 0.35**b**	13.08 ± 0.47**b**	20.88 ± 0.33**bc**	27.96 ± 0.37**bc**	33.88 ± 0.56**bc**	8.83 ± 0.39**ab**	19.19 ± 0.32**ab**	29.35 ± 0.46**cd**	38.36 ± 0.81**b**	
50	9.21 ± 0.23**a**	16.04 ± 0.24**a**	24.17 ± 0.28**a**	31.42 ± 0.31**a**	37.29 ± 0.32**a**	9.58 ± 0.39**a**	20.67 ± 0.14**a**	32.50 ± 0.45**ab**	41.86 ± 0.60**a**	
100	8.50 ± 0.37**a**	14.88 ± 0.44**a**	22.50 ± 0.66**ab**	30.13 ± 0.62**ab**	36.58 ± 0.65**ab**	9.96 ± 0.28**a**	21.04 ± 0.41**a**	33.25 ± 0.46**a**	40.11 ± 0.79**ab**	
200	6.92 ± 0.33**b**	12.96 ± 0.33**b**	20.18 ± 0.34**c**	27.00 ± 0.58**c**	32.92 ± 0.68**c**	9.71 ± 0.33**ab**	20.08 ± 0.87**ab**	31.00 ± 0.64**bc**	39.33 ± 0.22**ab**	
500	4.74 ± 0.07**c**	9.11 ± 0.57**c**	15.64 ± 0.38**d**	21.92 ± 0.51**d**	27.60 ± 0.60**d**	8.76 ± 0.27**ab**	17.28 ± 0.44**b**	27.63 ± 0.51**d**	35.69 ± 0.44**c**	
1000	4.08 ± 0.12**c**	5.50 ± 0.17**d**	9.79 ± 0.27**e**	13.50 ± 0.81**e**	18.72 ± 0.88**e**	7.46 ± 0.08**b**	14.04 ± 0.27**c**	21.46 ± 0.31**e**	27.92 ± 0.23**d**	

Interestingly, besides the growth differences observed on plates, the *F. graminearum* mycelia showed different color morphs dependent on the *(E)*-β-caryophyllene concentration (Table 4.1). Plates supporting the highest growth rate (50 and 100 ng μl^{-1}) turned a deep red color ten days post inoculation. As the *(E)*-β-caryophyllene concentration increased above 100 ng μl^{-1} or the media was devoid of *(E)*-β-caryophyllene, the color turned more yellowish. Based on the literature, this red pigment is likely to be aurofusarin, a group of naphthoquinone type of polyketides that is commonly produced by many *Fusarium* fungal species (Frandsen et al., 2006).

4.2.4 Overexpressing *(E)*-β-caryophyllene in Hi-II did not alter the levels of plant defense hormones

In order to understand how *(E)*-β-caryophyllene in Hi-II might act to increase fungal growth, we determined the levels of plant defense hormones in wild-type and *(E)*-β-caryophyllene-producing transgenic lines. SA and JA are synthesized by plants in response to challenge by various pathogens and are essential signaling molecules involved in both local and systemic acquired resistance (Bari & Jones, 2009). We found that overexpressing *(E)*-β-caryophyllene in Hi-II did not affect the baseline concentrations of either SA or JA (Fig. 4.3).

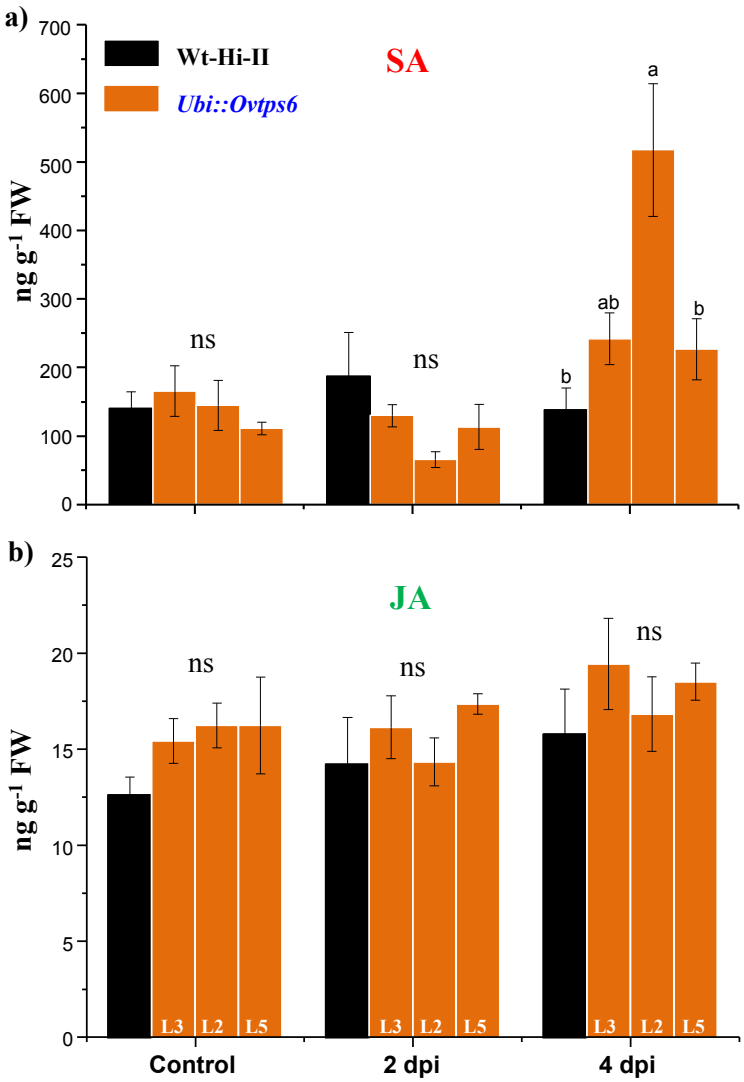

Fig. 4.3: *C. graminicola* induced phytohormone responses of wild-type and *(E)*-β-caryophyllene over-producing Hi-II lines. Fifteen days-old plants were inoculated with $1X10^{-5}$ *C. graminicola* spore concentrations. (**a**) SA and (**b**) JA were extracted and analyzed at 2 and 4 days post inoculation (dpi). Bars with different letters represent significant differences at α = 0.05 after ANOVA followed by Tukey HSD analysis.

However, we observed a trend towards increased JA in the transgenic lines especially at later time points though it was not statistically significant (Fig. 4.3b). The concentration of SA did not change two days post inoculation with *C. graminicola* spores compared to the mock-treated control plants in all plant genotypes analyzed (Fig. 4.3a). However, at four days post-inoculation, the level of SA was induced significantly in all *(E)*-β-caryophyllene producing transgenic lines. The level of JA however was unaffected at both two and four days post inoculation with *C. graminicola* (Fig. 4.3b).

4.2.5 *(E)*-β-caryophyllene expression did not alter the expression of maize defense genes after *C. graminicola* infection

To explore how *(E)*-β-caryophyllene might increase maize susceptibility to *C. graminicola,* we compared expression of some pathogen defense genes in plants producing *(E)*-β-caryophyllene and controls after infection. Expression of the pathogen resistance gene *ZmPR10* did not change significantly after two days post inoculation (Fig. 4.4a). The transcript accumulation was increased 3 to 4 fold after four days compared to the mock treated control plants. However, no statistical difference was observed between the wild-type and the *(E)*-β-caryophyllene-expressing transgenic lines. A similar pattern of expression was observed for *ZmBx7*, a gene involved in the biosynthesis of benzoxazinoids, a group of anti-fungal and anti-herbivore defenses. At two days post inoculation, the expression of *Bx7* did not change significantly from control levels, but at four days post inoculation it increased. However, there were no significant differences in the transcript levels of *ZmBx7* in the *(E)*-β-caryophyllene-emitting lines compared to the wild-type HI-II, except for one line (Fig. 4.4b). Interestingly, the maize actin gene was found to be four-fold induced in the two high *(E)*-β-caryophyllene emitting transgenic lines, 202-L2 and 202-L5 (Fig. 4.4c).

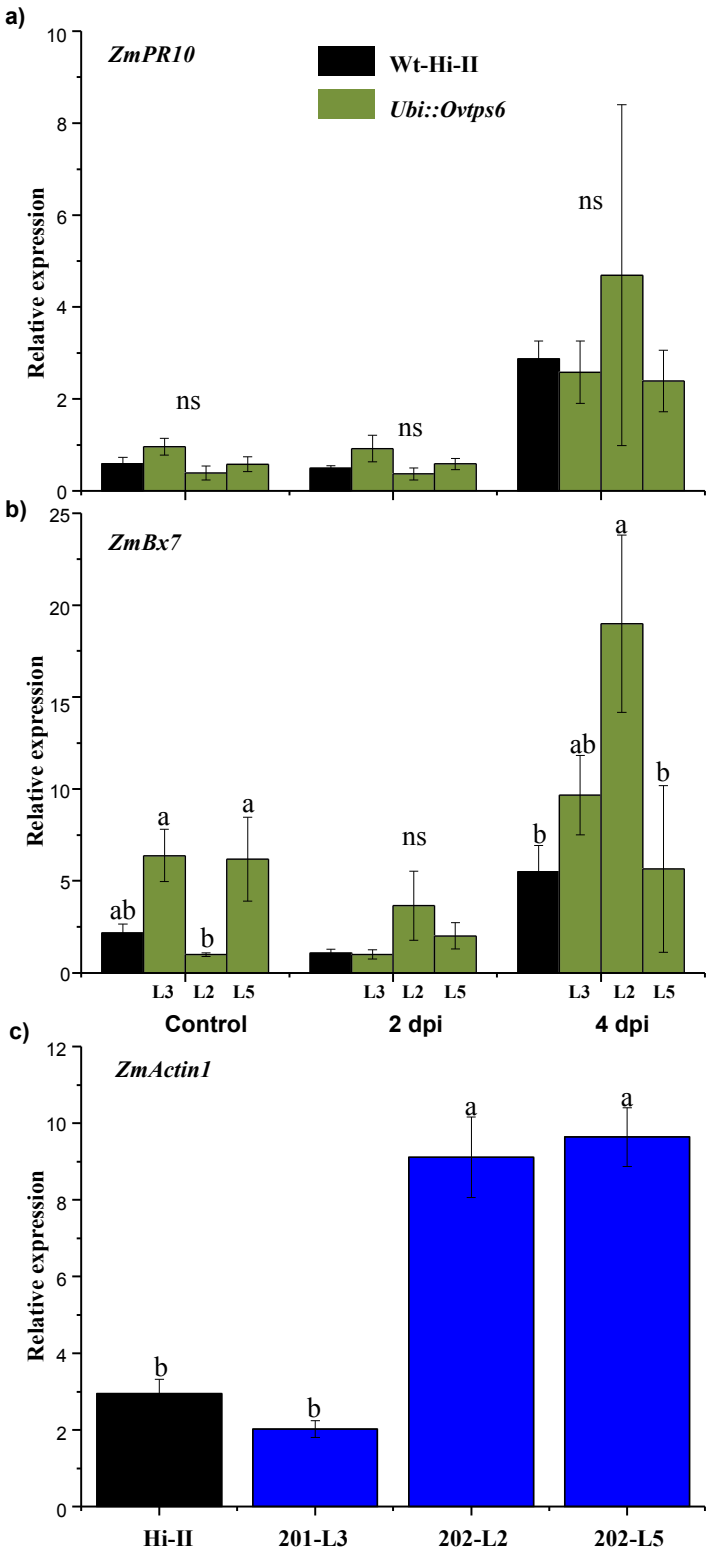

Fig. 4.4: *C. graminicola*-induced gene expression analysis. Fifteen days-old plants were inoculated with $1X10^{-5}$ *C. graminicola* spores. Samples were collected after 2 and 4 days post inoculation (dpi). Third and fourth leaves were pooled for the RNA extraction. Gene expression was conducted on five biological replicates using *RPB1* as normalizer gene. (**a**) *ZmPR10* and (**b**) *ZmBx7* were analyzed at both time points, but (**c**) *ZmActin1* was analyzed at 4 dpi. Bars with different letters represent significant differences after ANOVA followed by Tukey HSD test at $\alpha = 0.05$.

4.2.6 *C. graminicola* infection decreases glucoside conjugates of benzoxasinoids in the transgenic lines

One of the first defensive barriers that pathogens encounter in maize is the presence of antimicrobial benzoxazinoids (Ahmed et al., 2011). Thus we compared the accumulations of benzoxazinoids in wild-type and *(E)*-β-caryophyllene-producing transgenic lines before and after *C. graminicola* inoculation. Expressing *(E)*-β-caryophyllene in Hi-II generally did not affect the baseline concentrations of two of the major benzoxazinoids, DIBOA-glc and DIMBOA-glc (Fig. 4.5), except in one case. However, 2 and 4 days after fungal infection benzoxazinoid levels in the *(E)*-β-caryophyllene-producing transgenic lines decreased to significantly lower levels than in the Hi-II wild-type controls.

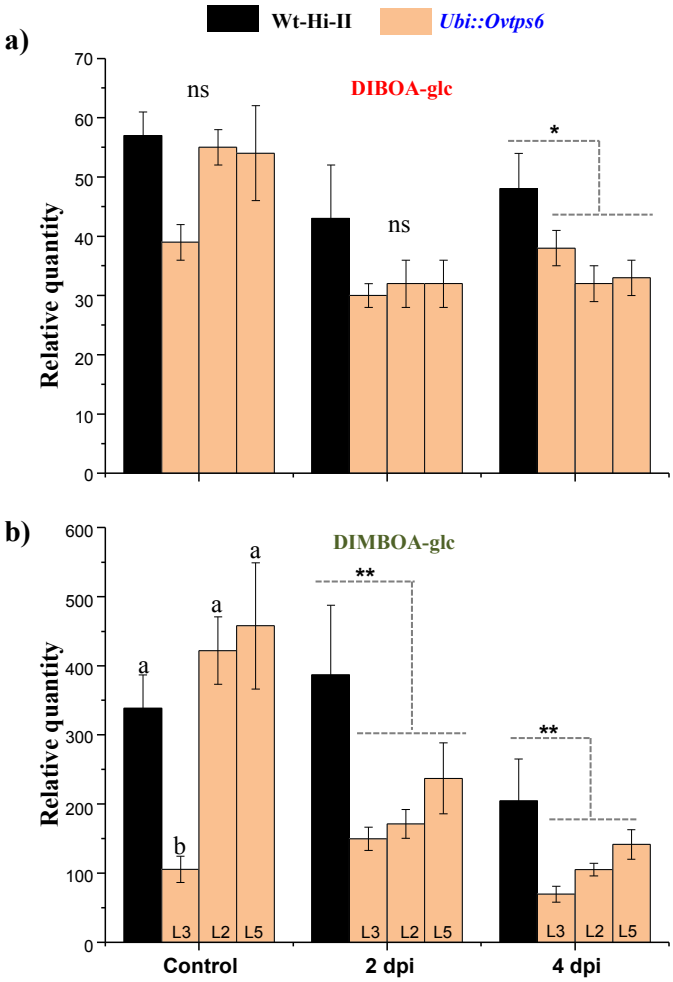

Fig. 4.5: Benzoxazinoids accumulation in *C. graminicola*-inoculated Hi-II maize lines. (**a**) DIBOA-glc and (**b**) DIMBOA-glc were compared in wild-type Hi-II and in the *(E)*-β-caryophyllene overproducing transgenic lines designated here as L3, L2, and L5 at 2 and 4 days post incoculation. Bars with different letters or asterisks show significant differences after ANOVA followed by Tukey HSD test at $\alpha = 0.05$.

4.3 Discussion

Maize cultivars differ in their ability to produce the volatile sesquiterpene (*E*)-β-caryophyllene in response to herbivory by *Diabrotica virgifera virgifera* (Rasmann et al. 2005; Degenhardt et al. 2009). Release of *(E)*-β-caryophyllene from roots attracts entomopathogenic nematodes that attack *D. v. virgifera* larvae and so reduces damage from this pest. However, most American maize lines, but not European lines, have lost their ability to produce *(E)*-β-caryophyllene during breeding (Degen et al. 2004; Köllner et al. 2008). It was previously shown that restoring the ability to produce *(E)*-β-caryophyllene in a Hi-II background increases maize resistance to *D. v. virgifera*. By contrast, in this study restoring *(E)*-β-caryophyllene production reduced maize resistance to infection by *C. graminicola*, suggesting an *(E)*-β-caryophyllene-based defense tradeoff against herbivores and pathogens.

Transgenic Hi-II maize lines with high *(E)*-β-caryophyllene emission rates developed massive anthracnose disease symptoms by seven days following inoculation with *C. graminicola* (Fig. 4.2a&b). These symptoms included anthocyanin pigmentation which has previously been shown to occur in several maize cultivars inoculated with anthracnose and other fungal pathogens (Hammerschmidt & Nicholson, 1977; Hipskind et al., 1996). This pigment was identified as a zwitterionic anthocyanin, cyanidin 3-dimalonylglucoside, and was suggested to act as a scavenger of cationic and anionic molecules that can be toxic for the plant. However, accumulation of this pigment does not mean that the plant is chemically well defended because *C. graminicola* has been shown to cause anthracnose disease in the presence of this pigment (Vargas et al. 2012). Using *C. graminicola actin* gene accumulation as a marker, we confirmed that transgenic high *(E)*-β-caryophyllene-producing lines contain the highest fungal biomass in their leaf tissue 4 dpi (Fig. 4.2c). Thus *(E)*-β-caryophyllene can decrease resistance to *C. graminicola*, and it is conceivable that the loss of *(E)*-β-caryophyllene emission from the North American maize lines may be due to selection by breeders for fungal resistance that inadvertently increased susceptibility to an insect herbivore. Recently, Erb et al (2011) compared the defense responses of selected American and European maize lines to insect and pathogen attack and correlated these to their volatile emission traits. Consistent with our results, most American maize lines that do not produce *(E)*-β-caryophyllene were found more resistant to the fungus *C. graminicola*. Interestingly, resistance to herbivores

and resistance to pathogens were negatively correlated inferring that adaptation of maize lines and their associated pests to the environmental conditions on different continents (Rebourg et al., 2003) might lead to a trade-off in resistance to pathogens and herbivores.

The hypothesis that *(E)*-β-caryophyllene production reduces maize resistance to pathogens is also well supported by the *in vitro* stimulatory effect of *(E)*-β-caryophyllene on the growth of *C. graminicola* and *F. graminearum* (Table 4.1). At concentration levels close to the naturally observable emission rates in maize, *(E)*-β-caryophyllene supports faster growth rates for both of the fungal pathogens investigated. However, higher concentrations of *(E)*-β-caryophyllene exhibit inhibitory effects on both fungal species. These results are consistent with the proposal that the fungicidal activity of terpenes and other essential oil components depends on different modes of activity at different concentrations. At low concentrations, fungicidal activity is variable and directly related to the characteristics of the individual compounds, whereas at higher concentrations fungicidal activity is less variant and is due to a common mechanism (Cavanagh, 2007). In our case, *(E)*-β-caryophyllene actually stimulates fungal growth at low concentration. The lack of reports on the stimulatory effects of volatile terpenoids on microbial growth is in striking contrast to the numerous studies on the antimicrobial properties of these substances (Rodov et al. 1995; Cavanagh, 2007; Huang et al. 2012; Fontana, PhD dissertation). However, our result is not the first to show stimulatory effects of terpenoid-based volatiles towards pythopathogenic fungi. For instance, wound induced citrus volatiles which are dominated by monoterpenes have been shown to function as cues for host recognition, germination and growth of *Penicillium digitatum* and *Penicillium italicum* (Droby et al. 2008). When terpene production in citrus plants was artificially blocked by genetically down-regulating terpene synthase activities the lack of terpenes led to increased resistance towards the fungus *P. digitatum* and the bacterium *Xanthomonas citri* (Rodriguez et al. 2011). In contrast to such specialist pathogens of citrus, non-host pathogens were either unaffected or inhibited by citrus volatiles (Droby et al. 2008; Rodriguez et al. 2011). The specific stimulatory effect of volatiles on citrus pathogens and inhibitory effect on non-pathogens indicate the possible role of these substances in host selectivity of some pathogens. Although we have not yet tested other non-pathogenic fungi of maize, we speculate that the stimulatory effect of *(E)*-β-caryophyllene might only be evident for host-specific pathogens.

It has been shown that in maize lines which differ in their production of *(E)*-β-caryophyllene or in transgenic Arabidopsis plants overexpressing a different group of sesquiterpenes, the baseline phytohormone levels are altered, which may lead to differential resistance to pests (Shivaji et al. 2010; Smith et al. 2012). However, overexpressing the oregano *(E)*-β-caryophyllene synthase gene in the non-producing maize line Hi-II did not affect the baseline concentration of either SA or JA (Fig. 4.3), two hormones that regulate plant responses to biotrophic and necrotrophic fungi, respectively (Lopez et al. 2008). But, at 4 dpi, a time at which *C. graminicola* is still supposed to be in a biotrophic stage, SA is found in higher concentrations in *(E)*-β-caryophyllene-producing transgenic lines (Fig. 4.3a). Although higher SA levels may be thought to be more effective in triggering systemic acquired resistance (SAR) response (Grant & Lamb, 2006; Bari & Jones, 2009), increased SA is not always an indicator of resistance. In fact, high SA levels may be a sign of sensitivity to pathogens (Rao et al. 1997; Rao & Davis, 1999).

It is well known that plants challenged by microbial pathogens express a set of defense-related genes following SA accumulation. The best known examples of these genes are the pathogenesis-related (PR) proteins. In addition to the *ZmPR10* gene that we analyzed (Fig. 4.4a), there are several other PR proteins which are induced after pathogen attack in different maize lines (Morris et al. 1998; Chen et al. 2010; Huffaker et al. 2011). Interestingly, an actin gene that we chose for the normalization of our qRT-PCR analysis was found to be also induced by pathogen attack, but only in the two transgenic lines with the highest *(E)*-β-caryophyllene emission (Fig. 4.4c). This is in agreement with other findings showing the induction of a plant actin gene expression during a compatible plant-pathogen interaction at the biotrophic phase (Jin et al. 1999). It is suggested that increased actin expression could be due to cytoskeleton rearrangement, a possible first line of defense in plant hosts in response to a biotrophic infection (Schmelzer, 2002). Altogether, this is another indication that *(E)*-β-caryophyllene producing transgenic lines were highly infected by the fungus.

In an effort to understand whether the levels of preformed defense compounds like benzoxazinoids are affected by the expression of *(E)*-β-caryophyllene, we compared benzoxazinoid accumulation in wild-type and transgenic lines. The concentration of the dominant benzoxazinoids, DIMBOA-glc and DIBOA-glc, did not differ between wild-

type and transgenic lines before fungal infection, suggesting that the transformation did not affect the biosynthesis of these compounds. Benzoxazinoids are synthesized in maize seedlings and stored as glucosides, which are then converted by glucosidases to the more biocidal aglucones upon tissue disruption by herbivore attack or pathogen infection (Niemeyer, 2009; Dafoe et al. 2011). Interestingly, after infection by *C. graminicola,* both DIMBOA-glc and DIBOA-glc were reduced significantly in the *(E)*-β-caryophyllene-producing transgenic lines (Fig. 4.5), indicating the higher degree of pathogen-caused tissue damage in these plants as compared to the controls. A reduction in DIMBOA-glc concentration was also reported in other studies following herbivore attack (Dafoe et al. 2011) or pathogen infection (Weibull & Niemeyer, 1995). Unfortunately, we could not detect the free aglycone forms of the benzoxazinoids which are often described as unstable (Cambier et al. 2000). Benzoxazinoids are generally thought to have little importance as antifungal agents for *C. graminicola* (Lyons & Nicholson, 1989). Hence, the conversion of DIMBOA-glc and DIBOA-glc to their respective aglucones may not help in the resistance of the *(E)*-β-caryophyllene emitting transgenic lines to the fungus. The high degree of glucose hydrolysis in the *(E)*-β-caryophyllene emitting lines is merely indirect evidence for the high degree of tissue damage by the fungus in these lines.

Maize inbred lines commonly grown in Europe and North America differ in their resistance to a root herbivore, *Diabrotica virgifera virgifera* (Rasmann et al. 2005; Degenhardt et al. 2009). This difference is attributed to genetic differences in the production of *(E)*-β-caryophyllene where most North American maize lines have lost their ability to produce it during breeding (Degen et al. 2004; Köllner et al. 2008). In this study we showed that restoring the ability to produce *(E)*-β-caryophyllene in a non-producing hybrid Hi-II compromised its resistance to *C. graminicola* infection, suggesting that the fungus may use *(E)*-β-caryophyllene as a signal for host recognition and successful colonization. It seems that the ability to produce *(E)*-β-caryophyllene leads to a trade-off in defenses against herbivores vs. defenses against pathogens. Thus it may not be surprising that maize lines bred in different locations vary in their potential to synthesize this sesquiterpene.

4.4 Materials and Methods

4.4.1 Plant and fungus material

The maize hybrid Hi-II (A188XB73) (Armstrong et al., 1991) which does not produce *(E)*-β-caryophyllene was transformed with the *Origanum vulgare (E)*-β-caryophyllene synthase gene under the control of the maize ubiquitin promoter (Degenhardt et al., 2009). Three independent selfed T_2 transgenic lines (201-L3, 202-L2 and 202-L5) were used in this experiment. As a nontransformed control, the selfed F_2 progeny of Hi-II was used. All the plants were maintained in a climate chamber with the following growth conditions: 300 µmol $m^{-2}s^{-1}$ PAR (photosynthetically active radiation), 60 percent RH (relative humidity), 24 ^{o}C/20 ^{o}C day/night temperature and 14 h photoperiod. Seeds were surface sterilized with 70 percent ethanol and sown in 10 cm diameter pots filled with commercial potting soil.

Colletotrichum graminicola strain M1.001 (kindly provided by Steffen Münch, Martin Luther University of Halle, Germany) was used as a foliar pathogen for disease development assessment in the different maize genotypes. The fungus was maintained on oatmeal agar (OMA) with continuous illumination from a white fluorescent light source at 25 ^{o}C for spore generation. Conidia were harvested from OMA plates with *C. graminicola* culture which were grown for 10-20 days. The conidia were counted with a hemocytometer and spore suspensions were adjusted to a concentration of 1 X 10^5 conidia ml^{-1}. Before leaf inoculation, 0.02 percent Tween 20 was added to the conidial solution as a surfactant and groups of five 15-day old maize seedlings were sprayed with 10 ml of the suspension. For the *in vitro* anti-fungal assay, *C. graminicola* and *Fusarium graminearum* (FSU59, Jena microbial resource collection) were grown on potato dextrose agar medium (PDA, Difco Laboratories, Detroit, MI) supplemented with different concentrations of *(E)*-β-caryophyllene.

4.4.2 Plant volatile collection and analysis

A dynamic headspace volatile collection system, which was installed in a climate-controlled chamber (24/20 ^{o}C day/night temperature, 60 percent relative humidity, 300 µmol $m^{-2}s^{-1}$ photosynthetically active radiation, 14/10 h light/dark condition) was employed to collect volatiles from wild-type and transgenic maize lines. In brief, ten-day

old maize plants were placed independently in a 3 l glass cylinder (Schott, Jena, Germany) which has air inlet and outlet valves on the lid of the cylinder. Charcoal-filtered air was allowed to enter the cylinder with an adjusted flow rate of 2 l min^{-1} through Teflon tubing. After mixing with the volatile emitted from the plant, the air exited the cylinder through a volatile collection trap, 150 x 6.4 mm glass tube (Analytical Research Systems, Gainesville, FL, USA) containing 30 mg of Super Q (Alltech, Deerfield, IL) which was fixed inside the air outlet valve. All volatile collections were conducted during the light phase in a similar time frame for a period of 6 consecutive hours. After the collection, the volatiles trapped were eluted with 200 µl dichloromethane containing 5 ng μl^{-1} nonyl acetate as an internal standard. For the GC-MS and GC-FID analysis, we followed the same protocol described in section 3.4.2.

4.4.3 Real-time PCR monitoring of fungal development

Maize plants which were mock-treated or inoculated with *C. graminicola* spores were harvested at 0, 2, and 4 days post inoculation (dpi) and flash frozen in liquid nitrogen. DNA was extracted from 100 mg freshly ground tissue using the Qiagen DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA quality and concentration was determined spectrophotometrically with Nanodrop 2000C instrument (Thermo Fisher, Wilmington, DE, USA). Fungal growth in the plant tissue was monitored quantitatively by PCR using the *C. graminicola Actin* gene probe. Primer sets used for this analysis are listed in Table S9.3.1. The PCR was performed on a 50 ng plant gDNA template in a Stratagene Mx3000P Real-time PCR machine (La Jolla, CA, USA) using SYBR Green-based detection of dsDNA synthesis (Applied Biosystems, Darmstat, Germany). The relative quantity of fungal biomass in the plant tissue was estimated based on a standard cure generated using PCR reactions carried out with different concentrations of *C. graminicola* gDNA run on the same plate with the same set of primers.

4.4.4 Determination of antifungal activities of *(E)*-β-caryophyllene

In order to determine the effect of *(E)*-β-caryophyllene on the growth of *C. graminicola* and *F. graminearum*, an *in vitro* poisoned food assay (Grover & Moor, 1962) was employed. Briefly, freshly prepared fungal cultures of both species were punched aseptically with a sterile cork-borer of 7 mm diameter. The fungal plugs were then put on

the center of PDA plates containing the desired *(E)*-β-caryophyllene concentration. The PDA medium was first autoclaved and then cooled down to 50 ^{o}C in a water bath before adding *(E)*-β-caryophyllene. The desired concentrations of *(E)*-β-caryophyllene, prepared by dissolving various amounts of the pure compound in 1 ml dimethylsulfoxide (DMSO), were immediately mixed with 20 ml of the cooled PDA medium and poured into Petri plates. In the control plates, equal volumes of DMSO without *(E)*-β-caryophyllene were added to the PDA medium. The Petri dishes were incubated in the dark at 28 ^{o}C. Colony diameter was then measured daily until the mycelia reached the edge of the Petri dish.

4.4.5 Phytohormone extraction and analysis

For SA and JA determination, 15-day old plants were first inoculated with *C. graminicola* spores at a concentration of 1 X 10^5 conidia ml^{-1}. Samples were then collected 2 and 4 days post inoculation and immediately flash-frozen in liquid nitrogen and stored in minus 80 ^{o}C. Approximately 200 mg of frozen material were ground to a fine powder using a precooled mortar and pestle. The powder was then transferred into a 2 ml Eppendorf tube containing 500 mg FastPrep matrix (BIO 101, Vista, USA). To each sample 0.5 ml methanol spiked with 10 ng D_2-JA and D_4-SA as an internal standard was added and homogenized in a reciprocal shaker for 5 min. Samples were then vigorously vortexed for 5 min and centrifuged at 13,000 rpm for 20 min at 4 ^{o}C. Then supernatant, usually 300 µl, was collected in an HPLC vial. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies, Boeblingen, Germany) following the procedure described in section 2.3.2.

4.4.6 Gene expression analysis by qRT-PCR

Total RNA was isolated from *C. graminicola* inoculated and mock-treated maize leaves using the Qiagen RNeasy Plant Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. To remove residual genomic DNA, the extracts were treated with RNase-free DNase (Qiagen, Hilden, Germany). RNA quality was measured with an Agilent 2100 Bioanalyzer (Waldbronn, Germany) and quantified spectrophotometrically with a Nanodrop 2000C instrument. For cDNA synthesis, Superscript III reverse polymerase (Invitrogen, Carlsbad, USA) was applied to 2 µg total RNA following the manufacturer's instructions. The qRT-PCR was performed on 3-fold diluted cDNA templates in a Stratagene Mx3000P Real-time PCR machine (La Jolla, CA, USA) using

SYBR Green-based detection of dsDNA synthesis (Applied Biosystems, Darmstat, Germany). The threshold cycle (Ct) values of *ZmPR10* and *ZmBx7* were normalized to the housekeeping gene encoding the RNA Polymerase II Larger subunit (*RPB1*). This gene has been previously used as an endogenous control in maize (Köllner et al., 2010). All primer pairs used in the qRT-PCR analysis are listed in Table S9.3.1.

4.4.7 Quantification of benzoxazinoids

Benzoxazinoid extraction and analysis was adapted from Erb et al (2009). Briefly, the plant material was flash frozen in liquid nitrogen and ground to a fine powder using mortar and pestle. Approximately 100 mg of the powder was suspended in 1 ml extraction buffer (98 percent methanol, 2 percent acetic acid) and vortexed for 5 minutes. Then, the samples were centrifuged at 12,000 *g* for 10 min and 800 µl of the supernatant was collected in HPLC vials. Samples were diluted three-fold and 10 µl was injected for the HPLC analysis. Samples were analyzed with a UV detector (260 nm) on a Sphinx-Säule Column with pure water as a mobile phase. Since we did not have internal standards, we present the data as relative detector response.

5. General Discussion

Plants emit a substantial portion of their fixed carbon back into the atmosphere in the form of volatile organic compounds (VOCs). This process costs plants a large portion of fixed energy that could otherwise be allocated for growth, development and reproduction and thus volatiles should have important functions. Plant volatiles have been implicated in a range of ecological functions including direct defense against herbivores and pathogens (Bernasconi et al., 1998; Huang et al., 2012), the attraction of natural enemies of herbivores (Turlings & Wackers, 2004), the priming and elicitation of defenses against imminent attack (Engelberth et al., 2004), and the mitigation of oxidative stresses (Loreto et al., 2004).

Terpenoid volatiles are the major contributors to the diversity of VOCs and thus have been studied in several ecological experiments. Maize (*Zea mays*) has been used as a model in our laboratory to study the biochemical and molecular aspects of terpene formation with special focus on the sesquiterpene hydrocarbons. Accordingly, over 20 different terpene synthase genes which are responsible for the formation of more than 100 different terpene hydrocarbons have been identified (Köllner et al., 2004a&b). Over the past years transgenic Arabidopsis plants expressing some of these terpene synthase genes were used to investigate the ecological relevance of individual groups of terpene volatiles (Schnee et al., 2006, Fontana et al., 2011). However, most of these investigations were mainly designed to understand the importance of these volatiles in attracting natural enemies of herbivores. In this thesis we employed transgenic Arabidopsis and maize plants expressing various sesquiterpene synthase genes to investigate sesquiterpene volatiles in other roles: in priming and elicitation of direct defenses against herbivores (Chapter I), in plant protection against ozone stress (Chapter II), and in plant-fungus interactions (Chapter III).

5.1 Manipulation of sesquiterpenes in transgenic Arabidopsis

Due to the complexity of blends and difficulties in obtaining compounds with the correct chirality from commercial sources or chemical synthesis, it has not been possible to study the ecological roles of sesquiterpene blends by simple reconstitution. However,

molecular methods in which blends encoded by single sesquiterpene synthases can be over-expressed or knocked-out in plants may provide a good alternative. Creating knock-out plants for sesquiterpene volatile of interest has been reported in wild tobacco (Kessler et al., 2006), however, it is usually difficult to achieve knock-outs in monocots like maize (Schnee, Phd Dissertation). In addition, creating mutant maize lines lacking most of their volatiles would be complex to engineer. However, recent advances in transgenic plant systems have created other promising platforms to study the biosynthesis and ecological roles of terpene volatiles.

Transgenic Arabidopsis transformed with different terpene synthase genes has been reported to produce a range of terpene volatiles (Aharoni et al., 2006; Schnee et al., 2006; Fontana et al., 2011). Since Arabidopsis does not normally produce detectable levels of terpene volatiles at the rosette stage, this stage is particularly suitable to study the ecological functions of sesquiterpenes without background interference (Chapter I & II). In addition, there is ample knowledge on the biology of this model plant to facilitate the characterization of important traits that might be altered due to terpene expression or gene transformation.

Despite the frequent use of Arabidopsis for the ectopic expression of terpene volatiles, gene transformation by itself may sometimes cause unintended or pleiotropic effects in transgenic plants (Cellini et al., 2004; Deng et al., 2008). It is, therefore, essential to assess such non-target effects that may potentially bias interpretation of results of ecological experiments generated on transgenic plants. However, we did not observe any alteration in C:N ratios or chlorophyll content or change in other primary metabolites that share the same biosynthetic pathway with the sesquiterpenes suggesting an ample supply of the substrate farnesyl diphosphate in the transgenic plants (see section 9.1 Supplementary material; Schnee et al., 2006). Genetic engineering of terpenes might negatively impact plant growth and fitness not only due to reduced supply of precursors for primary metabolites but also due to toxicity of the resulting compounds to plant cells (Mahmoud & Croteau, 2002; Aharoni et al., 2003). However, we have not observed any change in the overall phenotype of the transgenic plants that indicate delayed growth and development. Therefore, the use of transgenic Arabidopsis plants expressing the different terpene synthase genes in our study provides an opportunity to investigate the roles of sesquiterpene volatiles in plant defenses against biotic and abiotic stresses.

5.2 Overexpressing sesquiterpenes elicited direct defenses in Arabidopsis

Plant volatiles have been intensively investigated for their role in direct and indirect defenses. More recently, volatiles have been also implicated as signals for plant-plant communications. However, the nature of the volatiles involved and the molecular mechanism by which volatiles activate defenses are all poorly known. Although herbivore-induced plant volatiles are usually mixtures of chemically different compounds, most of the investigations conducted in this regard have focused only on green leaf volatiles. Few studies have suggested the involvement of terpene volatiles in priming or eliciting direct and indirect defenses in plants (Arimura et al., 2000; Kessler et al., 2006; Muroi et al., 2011). In Chapter I of this thesis we found a group of sesquiterpene volatiles to have a priming and defense elicitation effect when they are genetically expressed in Arabidopsis.

Although several investigations have clearly showed plants to increase their resistance to herbivory when exposed to herbivore-induced volatiles emitted from neighboring plants under laboratory conditions, the ecological relevance of this form of interaction in nature has been questioned (Heil & Karban, 2010). One of the arguments usually raised in this regard is that a volatile signal that improves the resistance of a potential competitor is not likely to evolve without benefiting the emitter itself (Farmer, 2001; Heil & Karban, 2010). However, recent discoveries of the within-plant signaling roles of induced volatiles resolved many concerns that were not explained in the context of volatile mediated between-plant signaling (Frost et al., 2007; Heil & Karban, 2010). These discoveries suggest the need for care in applying volatile signals in nature. In Chapter I, we explored the roles of the test volatiles by genetically expressing the corresponding biosynthetic genes constitutively in Arabidopsis and subsequently looking for any change in defense phenotypes. This approach is supported by the fact that plant volatiles may only be transmitted over short distances between plants in nature and thus expression within plants may be a realistic test situation (Fig. S9.1.1).

Exposure to green leaf volatiles and general induced volatile blends activates the accumulation of oxylipin pathway metabolites and gene transcripts in many plant species (Engelberth et al., 2004; Frost et al., 2008). Additionally, volatile exposure can induce the transcript accumulation of antiherbivore defense genes (Ton et al., 2007; Peng et al.,

2011), increase proteinase inhibitor activities (Kessler et al., 2006), and depending on the plant species exposed may trigger the accumulation of specialized defense metabolites such as nicotine and extra-floral nectaries (Kessler et al., 2006; Heil & Kost, 2006). Our studies with sesquiterpene expressing transgenic Arabidopsis plants showed that the sesquiterpenes themselves had similar responses increasing the baseline jasmonate levels, the transcripts of VSP2 and TPI genes, the level of TPI activity and glucosinolate concentrations (Chapter I). Irrespective of the mechanism, all these results suggest that sesquiterpenes can indeed function as endogenous signals to mount antiherbivore defenses. Previously it has been reported that Arabidopsis plants exposed to herbivore-induced volatiles or treated with synthetic monoterpenes activate a suite of molecular and transcriptome responses associated with the jasmonate signaling pathway (Godard et al., 2008; Zhang et al., 2012).

Though it is now generally accepted that plant volatiles can prime or directly induce plant defenses, the molecular mechanism of volatile perception and the subsequent signal transduction events that upregulate defense genes and metabolites are still poorly understood. Recent advances have highlighted the involvement of calcium signaling and changes in membrane potential as early steps in the perception of volatile signals (Mafei et al., 2007; Zebelo et al., 2012). Exposing Arabidopsis to a variety of plant volatiles including terpenes has been shown to promote the transient increase in cytosolic calcium levels (Asai et al., 2009). An increase in the cytosolic calcium level is one of the key components required for the activation of phytohormones and defense genes (Arimura et al., 2000). A more recent investigation has also highlighted epigenetic factors in providing the molecular basis for priming and defense induction by volatiles (Kim & Felton, 2013). Supporting this notion, a recent study showed that maize plants exposed to conspecific herbivore-induced volatiles activate the expression of a trypsin proteinase inhibitor gene. Interestingly, a series of methylation sites on the promoter region of this gene has been found to be demethylated following volatile exposure (Ali et al., 2013). Given the lipophilic nature of the sesquiterpene volatiles studied and the special tendency of these compounds to interact with membrane components, the endogenously produced sesquiterpenes may trigger this molecular cascade.

5.3 Sesquiterpenes are protective perfumes against ozone stress

In addition to roles in biotic stresses, plant volatiles are also implicated to have a function in protecting plants against abiotic stresses caused by high temperature, UV radiation and ozone exposure. Although there have been many field and laboratory studies of plant volatiles in this regard, most have focused only on isoprene (C_5) and a few monoterpenes (C_{10}). Sesquiterpenes (C_{15}) are emitted from large number of plant species, yet their role is less investigated in abiotic stress tolerance. Due to their high reactivity towards ozone and their greater aerosol formation potentials, sesquiterpenes are especially worth investigating (Winterhalter et al., 2009). In Chapter II of this thesis we studied the roles of selected maize sesquiterpenes in plant protection against ozone-induced stresses by genetically transforming Arabidopsis with the respective sesquiterpene biosynthetic genes.

In several investigations, isoprene has been suggested to react and quench ozone at the leaf boundary layer to levels that are less or non-toxic for plants (Loreto et al., 2001; Loreto & Fares, 2007). However, many less volatile monoterpenes and sesquiterpenes are proposed to be more reactive with ozone than isoprene in the atmosphere (Atkinson & Arey, 2003; Bonn & Moortgat, 2003; Fares et al., 2008). Given the higher reactivity of *(E)*-β-caryophyllene and *(E)*-β-farnesene with ozone, the protective effect observed (Chapter II) could be due to the efficient cleansing of ozone on the leaf surface of the transgenic plants before it is actually taken up by stomata. By acting as effective ozone sinks within canopies of Amazonian forests, sesquiterpene volatiles have been also suggested to reduce the harmful ozone uptake and its associated oxidative damage to plants (Jardine et al., 2011).

Exposure to ozone has been described to induce the emission of terpenoid and other types of volatiles in several plant species (Bourtsoukidis et al., 2012; Rinnan et al., 2005). As a result, plant species or cultivars which activate the emission of their terpenoid volatiles faster and more strongly have been shown to be better protected from ozone-induced oxidative stresses (Heiden et al., 1990; Pellegrini et al., 2012). These observations suggest that, like isoprene, sesquiterpene volatiles could be considered as defensive traits against ozone and other abiotic stresses. Thus, plant genotypes with

greater capacity for terpene volatile production and emission may have better fitness under ozone stress.

In addition to purging ozone on the leaf surface, solubilized or gas-phase terpenes with antioxidant properties may also remove stress-generated toxic reactive species inside plant tissues (Sharkey et al., 2008; Vickers et al., 2009). This has been shown in many plant species producing isoprene or monoterpenes by chemically inhibiting the biosynthesis of these volatiles with fosmidomycin and subsequently treating the plants with Rose Bengal (RB) to generate the reactive oxygen species (Affek & Yakir, 2002; Velikova et al., 2004): blocking the synthesis of these volatiles resulted in the accumulation of more singlet oxygen, H_2O_2 and MDA after RB treatment. Although there are analytical challenges to proving sesquiterpene reactions with reactive oxygen species, the reduction in such species in our transgenic plants could be partially explained due to the direct removal of these toxic compounds by endogenous sesquiterpenes (Chapter II).

Terpene volatiles have been proposed to directly interact with membrane components and enhance lipid-lipid and lipid-protein interactions hence stabilizing membrane under heat stress (Loreto et al., 1998; Sharkey et al., 2008; Chen et al., 2009; Vickers et al., 2009; Possell & Loreto, 2013). Direct evidence showing that isoprene stabilizes membrane lipids and reduces heat-induced phase transitions has been reported by Siwko et al (2007) using an *in vitro* molecular dynamics technique. Given their lipophilic nature, the sesquiterpene volatiles may also protect membranes from damage caused by ozone exposure by a similar mechanism.

Since the increase in tropospheric ozone concentration is a recent phenomenon, it is unlikely that terpene volatiles evolved as a mechanism to deal specifically with ozone toxicity. However, these compounds have been proposed to be a general coping mechanism against oxidative stresses caused by many other abiotic factors (Vickers et al., 2009). Depending on plant genotype and environmental condition, the current tropospheric ozone levels cause a substantial amount of yield reductions in many agricultural crops (Wilkinsons et al., 2012). Given the projected global population growth estimate from 6 to 9 billion in the year 2050 (Avnery et al., 2011), a continuously increasing background level of ozone may pose greater challenges in sustaining this population size. Therefore, we suggest that plants having traits for the production of

terpenoid volatiles and efficient antioxidant mechanisms may have a better chance of survival under future ozone levels.

5.4 Tradeoffs with sesquiterpene production and defense against herbivores and pathogens

Based on their genetic variability for the induced production of volatiles, plants exhibit pronounced differences in their resistance towards herbivore enemies. For instance, it has been reported that different maize inbred lines that show genetic variability on the emission of the sesquiterpene *(E)*-β-caryophyllene have a marked difference in their resistance towards herbivore enemies (Degen et al., 2004; Rasmann et al., 2005; Köllner et al., 2008; Degenhardt et al., 2009; Shivaji et al., 2010; Smith et al., 2012). Yet information on the impact of this variability on the resistance of these lines against microbial pathogens is limited. By genetically restoring the *(E)*-β-caryophyllene production in a non-producing maize line, we investigated the response of the plant towards the fungus *C. graminicola* (Chapter III).

Although volatiles are generally believed to be defensive against many potential enemies of plants including microbial pathogens (Huang et al., 2012), in the current investigation the sesquiterpene volatile tested was found to have an opposite effect: rather than defending the plant, *(E)*-β-caryophyllene becomes a signal for successful colonization by the fungus (Chapter III). This result was also supported by an *in vitro* antifungal assay where *(E)*-β-caryophyllene was found to stimulate hyphal growth of *Colletotrichum graminicola* and *Fussarium graminearum*. Consistent with our result, most maize genotypes which produce *(E)*-β-caryophyllene were found more susceptible to the fungus *C. graminicola* (Erb et al., 2011). Investigations in other plant species also showed a similar stimulatory effect of terpenoid-based volatiles towards other phythopathogenic fungi. For instance, wound-induced citrus volatiles dominated by terpenoids have been shown to function as cues for host recognition, germination and growth of *Penicillium digitatum* and *P. italicum* (Droby et al., 2008). Surprisingly, when terpene production was genetically down-regulated, citrus plants became more resistant to the fungus *P. digitatum* (Rodriguez et al., 2011). Therefore, many fungal pathogens may have developed counter-adaptive mechanisms to these defensive compounds and have even exploited them for their own benefit in host selection.

The genetic restoration of the *(E)*-β-caryophyllene signal in Hi-II maize line did not alter the natural antifungal defenses of the plant (Chapter III); instead the signal seems to increase the apparency of the plant to the fungus. *(E)*-β-caryophyllene has been also reported to increase the apparency of plants to adults and larvae of the herbivores *Spodoptera frugiperda* and *Diabrotica virgifera virgifera* (Hammack, 2001; Robert et al., 2013). However, since this compound is recruited for the attraction of natural enemies of the above herbivores, in environments where the natural enemy exists, the plants sustain less herbivore damage due to the orchestration of indirect defenses (Robert et al., 2013). Therefore, we suggest that maize inbred lines which retain the *(E)*-β-caryophyllene signal may encounter a defense tradeoff when the plants are exposed to herbivores and pathogens at the same time. Supporting this notion, resistance to herbivores and resistance to pathogens have been shown to be negatively correlated in maize inbred lines which are genetically variable in their volatile production (Erb et al., 2011).

Therefore, the loss of the *(E)*-β-caryophyllene signal in North American maize lines could have happened by chance during breeding for selecting desirable agronomic traits to resist the fungus. Alternately, the emission of the signal might be advantageous for the fungus to develop under North American environmental conditions and therefore this trait may have been selected against. The loss of the signal was specifically proposed to be due to the reduction in the transcription of the *tps23* gene, the gene responsible for the biosynthesis of *(E)*-β-caryophyllene (Köllner et al., 2008). We suggest that ceasing the production of *(E)*-β-caryophyllene by the North American maize genotypes may save resources that could be otherwise invested in other defenses. This suggestion is supported by recent evidence that maize plants in which *(E)*-β-caryophyllene production is restored suffer comparative declines in seed germination, plant growth and yield (Robert et al., 2013). Thus, although genetically manipulating plants for volatile emission might be a promising tool to enhance the resistance of plants against herbivore enemies, caution must be taken to check the consequences of this modification towards other plant pests.

6. Summary

Plants produce and release a diverse array of volatile organic compounds to their surroundings. This process costs plants a large amount of their fixed energy that could otherwise be allocated for growth, development and reproduction. However, volatiles are assumed to benefit plants through positive and negative interactions in the environment. Plant volatiles, for instance, attract pollinators and seed dispersers, protect plants directly from attacking herbivores and pathogens, entice predators and parasitoids that prey on herbivores, prime defenses against imminent attack, and mitigate oxidative stresses. Although plant volatiles are chemically highly variable, the focus of this thesis was to investigate the roles of volatile sesquiterpenes in defense against biotic and abiotic stresses by transforming selected sesquiterpene synthase genes in the background of a plant that does not produce the volatiles in question.

Hence, the first question addressed in this thesis was to investigate whether genetically enhancing the production of sesquiterpenes in Arabidopsis affects the performance of herbivores on the plant. To achieve this goal we overexpressed two maize sesquiterpene synthase genes, *Zm*TPS4 and *Zm*TPS5, in Arabidopsis and subsequently assessed the performance of larvae of the generalist herbivore *Spodoptera littoralis* on these transgenic lines. The results showed that the larvae performed poorly on sesquiterpene-emitting transgenic lines. However, this poor performance was not due to a direct olfactory deterrent effect of the sesquiterpenes themselves. Instead, the sesquiterpenes were found to elicit the further induction of defenses already present in Arabidopsis including glucosinolates and proteinase inhibitors. This defense elicitation was accompanied by increased baseline jasmonate concentration and increased transcripts of selected defense genes following herbivory. These results suggest that sesquiterpenes which are often emitted in response to herbivory can function as signals to prime the induction of anti-herbivore defenses.

The second research question addressed in this thesis was conceived based on the existing evidence that terpene volatiles such as isoprene and a few monoterpenes protect plants from oxidative stresses caused by ozone exposure. Sesquiterpene volatiles share many of the same chemical properties as isoprene and monoterpenes, yet their role in

plant protection against ozone damage is poorly investigated. Although few sesquiterpene volatiles are known to react and degrade ozone, this interaction has not been investigated from a phytocentric perspective. Therefore, in *Chapter II* of this thesis we investigated whether volatile sesquiterpenes protect plants from damages caused by ozone exposure by overexpressing two particular maize sesquiterpene synthases, *Zm*TPS10 and *Zm*TPS23, in Arabidopsis that are responsible for the formation of *(E)*-β-caryophyllene and *(E)*-β-farnesene sesquiterpenes respectively.

As a result, transgenic Arabidopsis plants overexpressing the above sesquiterpene volatiles were found to be better protected from ozone injury and displayed less visible injury, reduced ion leakage, higher photosynthesis rate and yield. The protective mechanism could be attributed to the ozone quenching abilities of the sesquiterpenes at the leaf boundary layer, which was shown by a 67 to 79 percent ozone degradation in the headspace of the transgenic lines. A similar degree of ozone cleansing was observed when authentic *(E)*-β-caryophyllene and *(E)*-β-farnesene standards were tested in chambers containing known concentrations of ozone *in vitro*. Sesquiterpenes may therefore protect plants against ozone damage by a mechanism similar to that of isoprene. However, since sesquiterpenes are emitted from more diverse plant species than isoprene, these compounds might have a greater ecological relevance for adaptation to ozone stress.

Previously it has been shown that genetically restoring the ability to synthesize *(E)*-β-caryophyllene in a non-producing maize line (Hi-II) improves its resistance against herbivores by attracting the natural enemies of the herbivores. However, it is largely unknown whether this modification affects the resistance of the plant to other pests. Therefore, in the last chapter of my thesis I investigated the response of this same transgenic maize line to infection by the fungus *Colletotrichum graminicola*. The results surprisingly showed that restoring *(E)*-β-caryophyllene in Hi-II increased the susceptibility of the plant to the fungus. This genetic modification did not alter the background levels of the plant's natural defenses including phytohormones, anti-fungal defense genes and metabolites. Instead, the expression of *(E)*-β-caryophyllene seemed to directly stimulate fungal growth in the plant. In an *in vitro* antifungal assay, we found out that *(E)*-β-caryophyllene stimulated hyphal growth of *C. graminicola* and *Fusarium graminearum*. Thus, although restoring *(E)*-β-caryophyllene production may improve the resistance of the plant against herbivores, it may compromise its resistance to major

fungal pathogens. This compound appears to be employed as host-finding cue by *C. graminicola* and *F. graminearum* for successful colonization of maize and this might explain its loss during maize breeding in environments where such pathogens are prevalent.

7. Zusammenfassung

Pflanzen produzieren und emittieren eine Vielzahl an Duftstoffen. Obwohl die Biosynthese dieser Stoffe sehr kostenintensiv ist, können Pflanzen in entscheidendem Maße davon profitieren. Duftstoffe dienen beispielsweise zur Anlockung von Bestäubern oder Samenverteilern. Sie können jedoch auch in der Pflanzenverteidigung eine Rolle spielen indem sie z.B. Fressfeinde abschrecken oder natürliche Feinde der Fressfeinde anlocken. Weiterhin können sie als Signalstoffe in der Pflanzenkommunikation wirken oder auch einen Schutz vor oxidativen Stress und Hitze darstellen. Im Rahmen dieser Arbeit sollte die Funktion von flüchtigen Sesquiterpenen in der Verteidigung der Pflanze gegen biotische und abiotische Stressfaktoren untersucht werden.

Als erstes wurde die Frage untersucht, ob heterolog in *Arabidopsis thaliana* produzierte Sesquiterpene einen Einfluss auf Herbivoren haben. Dazu wurden die Sesquiterpensynthasegene *tps4* und *tps5* aus Mais in Arabidopsis eingebracht und die Larvenentwicklung des generalistischen Herbivors *Spodoptera littoralis* an den transgenen Pflanzen untersucht. Die Ergebnisse zeigten, dass sich die Larven an den transgenen, Sesquiterpen-produzierenden Pflanzen schlechter entwickelten als an Kontrollpflanzen. Weiterführende Experimente ergaben, dass dieser Effekt nicht durch die direkte Wirkung der Terpene hervorgerufen wurde, sondern eher durch eine allgemeine Erhöhung der Pflanzenabwehr in den transgenen Pflanzen erklärt werden kann. Wahrscheinlich wirken flüchtige Sesquiterpene, die häufig als Duftstoffe nach Herbivorie abgegeben werden, als Signale für die Induktion bzw. das *Priming* von Abwehrreaktionen in anderen Pflanzenteilen oder benachbarten Pflanzen.

Die zweite Fragestellung dieser Arbeit ergab sich aus der Tatsache, dass einige Hemi- und Monoterpene Pflanzen vor oxidativen Stress, hervorgerufen durch z.B. hohe Ozonkonzentrationen, schützen können. Obwohl Sesquiterpene ähnliche physikochemische Eigenschaften wie Hemi- und Monoterpene besitzen und teilweise auch mit Ozon reagieren können, war bis jetzt unklar, inwieweit sie ebenfalls antioxidativ wirken. Im zweiten Kapitel dieser Arbeit wurden daher transgene Arabidopsispflanzen erzeugt, welche die Mais-Terpensynthasegene *tps10* und *tps23* überexprimierten und somit die Sesquiterpene (*E*)-α-Bergamoten und (*E*)-β-Farnesen bzw. (*E*)-β-Caryophyllen

produzierten. Diese Pflanzen wurden mit Ozon begast und auf ihre Ozontoleranz hin getestet. Es zeigte sich, dass die transgenen Pflanzen weniger oxidativem Stress ausgesetzt waren als die Kontrollpflanzen. Messungen ergaben eine um etwa 67-79 prozent verringerte Ozonkonzentration im Luftraum um die transgenen, Terpene-produzierenden Pflanzen. Ähnliche Ozonabbauraten konnten auch durch das Einbringen der reinen Testsubstanzen in die Messkammern erzeugt werden. Dies lässt darauf schließen, dass die hier getesteten Sesquiterpene (*E*)-α-Bergamoten, (*E*)-β-Farnesen und (*E*)-β-Caryophyllen mit Ozon reagieren und damit die Pflanze vor oxidativem Stress schützen können.

Kürzlich konnte gezeigt werden, dass gentechnisch-veränderte, (*E*)-β-Caryophyllen-produzierende Maispflanzen besser vor Herbivorie geschützt sind, da sie durch die Freisetzung des gebildeten (*E*)-β-Caryophyllen effektiv die natürlichen Feinde ihrer Herbivoren anlocken können. Im dritten Teil dieser Arbeit sollte nun untersucht werden, ob durch diese gentechnische Modifikation andere Abwehrmechanismen der Pflanze beeinflusst waren. Dafür wurden transgene Maispflanzen mit dem Pilz *Colletotrichum graminicola* infiziert und ihre Antwort auf den Pilzbefall gemessen. Obwohl die (*E*)-β-Caryophyllenproduzierenden Maispflanzen keine Veränderungen in den natürlichen Verteidigungsreaktionen zeigten, wiesen sie im Vergleich zu Kontrollpflanzen eine erhöhte Anfälligkeit für *C. graminicola* auf. *In vitro*- Assays mit reinem (*E*)-β-Caryophyllen zeigten, dass *C. graminicola*, aber auch *Fusarium graminearum*, mit einem verstärkten Hyphenwachstum auf dieses Terpen reagieren. Man kann spekulieren, dass (*E*)-β-Caryophyllen *in vivo* ein natürliches Signal darstellt, dass die Pilze benutzen können um ihre Wirtspflanzen zu finden.

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9. Supplementary Material

9.1 Supplementary material for Research Chapter I

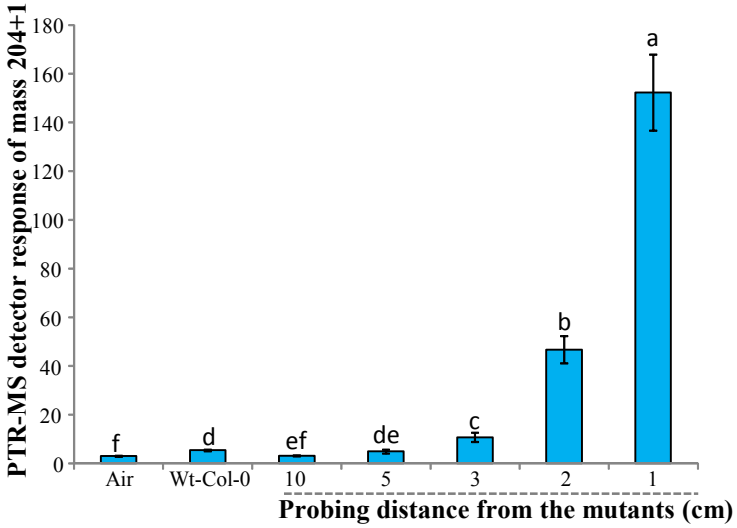

Fig. S9.1.1: Detectability of sesquiterpene volatiles in the headspace of the transgenic plants. Air samples were taken by a PTR-MS sampler unit from the head space of the sesquiterpene producing transgenic plants at various distances. The protonated molecular mass of 205 for sesquiterpenes were taken from the detector response for comparison. For comparison, background air and volatiles from the headspace of wild-type Col-0 at 1 cm distance were also analyzed. A similar cycle number was used for the sample probing time and the graphs represent the averages of the detector responses. Bars represent mean ± SE of 4 to 32 replicates. Different letters on each bar represent significant differences after ANOVA ($F_{6,72} = 206.46$, $p < 0.001$) followed by Tukey HSD post hoc test at $\alpha = 0.05$.

Table S9.1.1: Primer pairs used for the gene expression analysis

Gene name	AGI No.	Primer sequences 5'- 3'	Size
LOX2	At3g45140	Forward: AAGGATGGTGGAGTGAAGTG Reverse: TTGTCGTTGGTCGGTTGG	195
AOS	At5g42650	Forward: TTACGGCTCAATACGGTAG Reverse: GCTTCTCTCCTTCTTCTCC	183
VSP2	At5g24770	Forward: CACTTCTCTTGCTCTTGGC Reverse: CAGTTGGCGTAGTTGATGG	136
ATTI3	At2g43530	Forward: TTATCCGTCGTTGTGTTATCG Reverse: TTGACTATTACTTGTTCGTTTACC	194
ATTI6	At2g43550	Forward: GTGGTTGAGTATGGAGGAG Reverse: TTACAGTAGTCGCAGAAGC	161
ACTIN8	At1g49240	Forward: ATGAAGATTAAGGTCGTGGCAC Reverse: GTTTTTATCCGAGTTTGAAGAGGC	400

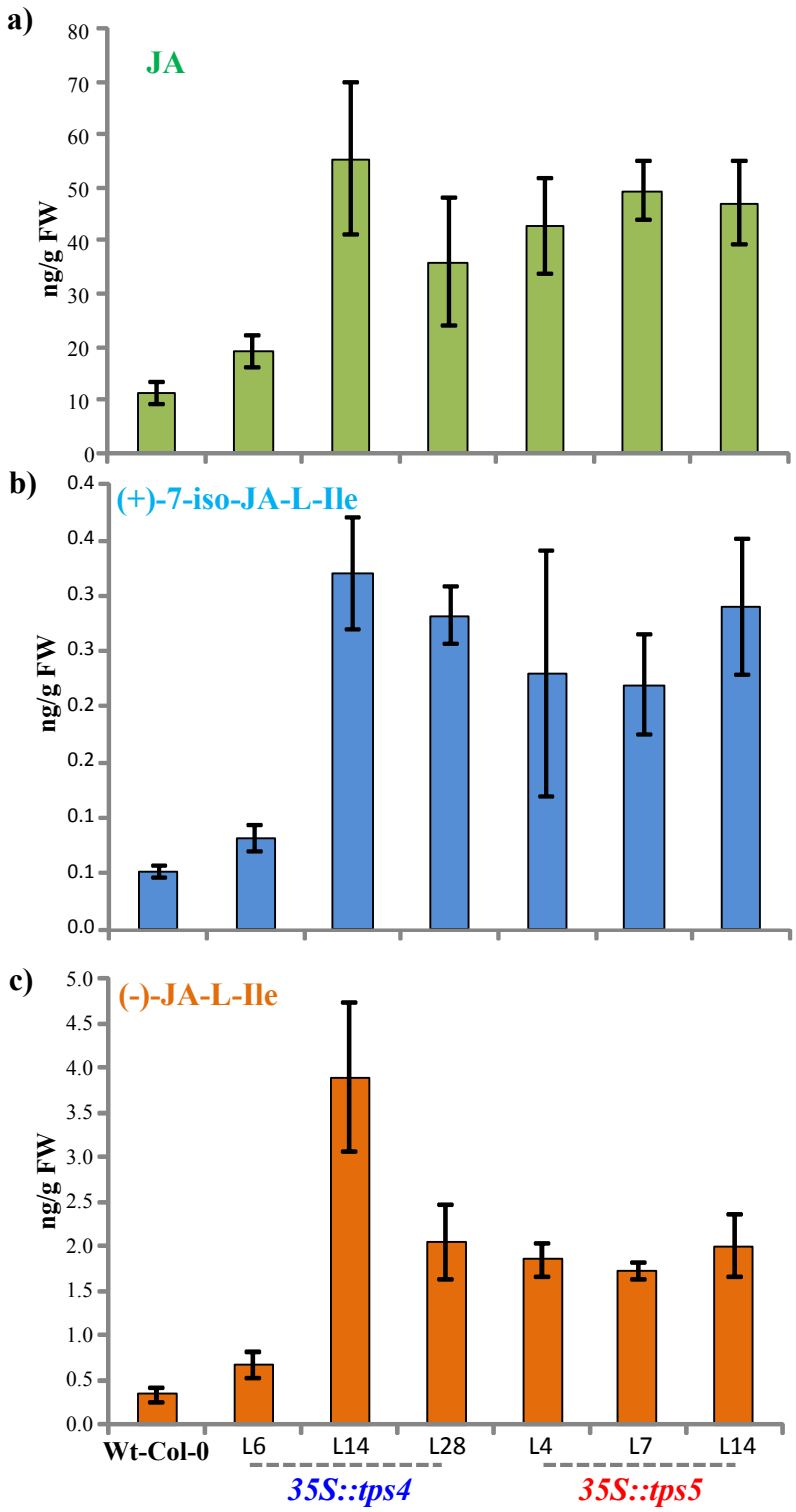

Fig. S9.1.2: Comparison of baseline jasmonate concentrations in wild-type and sesquiterpene overproducing transgenic lines. Uninduced rosette leaves of five week-old plants were harvested and flash-frozen in liquid nitrogen. The Jasmonates were extracted from 200 mg ground fresh weight with pure methanol containing 10 ng D2-JA and 2 ng JA-13C_6-Ile as internal standards. The extract was analyzed with a triple quad LC/MS/MS instrument.

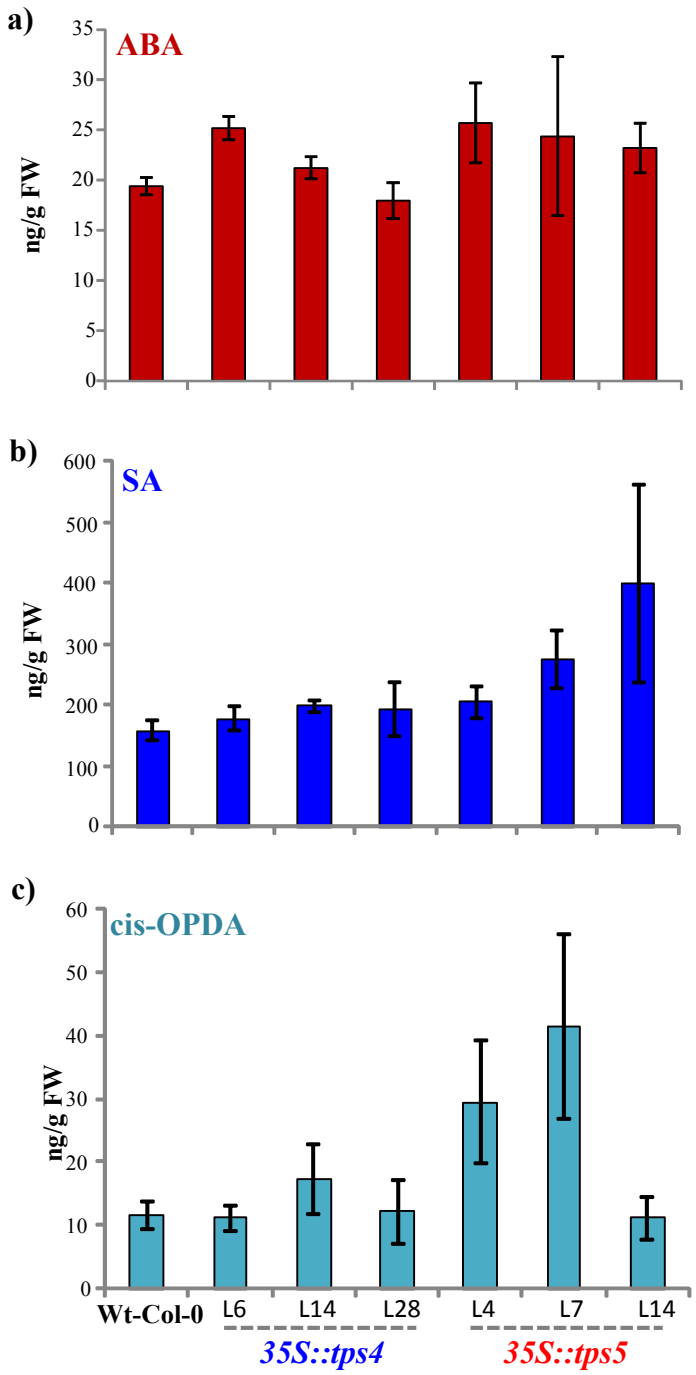

Fig. S9.1.3: Comparison of baseline jasmonate concentrations in wild-type and sesquiterpene overproducing transgenic lines. Uninduced rosette leaves of five week-old plants were harvested and flash-frozen in liquid nitrogen. The phytohormones were extracted from 200 mg ground fresh weight with pure methanol containing 10 ng of D_6-ABA, D_4-SA and D_2-JA as the respective internal standards. The extract was analyzed with a triple quad LC/MS/MS instrument.

Table S9.1.2: *Spodoptera littoralis* larval performance on sesquiterpene-emitting transgenic vs. non-emitting wild-type *A. thaliana* lines

Developmental parameter	N	Col-0	*35S::tps4*			*35S::tps5*		
			L6	L14	L28	L4	L7	L14
Time to pupation (days)	20	17.11 ±0.11	17.40 ±0.68	18.00 ±0.52	18.00 ±0.00	17.67 ±0.33	17.33 ±0.33	16.50 ±0.50
Pupation success (%)	20	75	30	50	10	40	25	40
Pupal weight (mg)	20	392.13 ±17.26	349.36 ±35.83	349.93 ±12.11	379.65 ±57.35	402.85 ±24.16	405.57 ±23.90	362.40 ±31.06
Time to adult emergence (days)	20	34.43 ±0.61	35.00 ±0.41	37.00 ±0.89	37.00 ±0.00	36.00 ±1.14	35.33 ±0.67	34.00 ±0.58
Adult emergence success (%)	20	35	20	15	5	15	15	15
Adult weight at emergence (mg)	20	247.25 ±18.23	229.71 ±37.36	212.01 ±11.60	192 ±0.00	245.64 ±22.48	242.05 ±31.05	240.05 ±10.90

Note: N represents only the total number of larvae used at the beginning of the experiment.

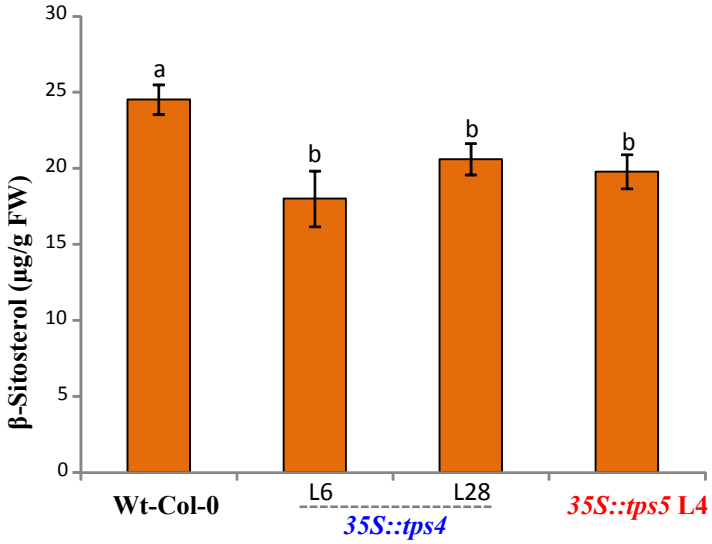

Fig. S9.1.4: Comparison of β-sitosterol concentrations in wild-type and sesquiterpene producing transgenic lines. Rossette leaves of five week-old plants were harvested and flash-frozen in liquid nitrogen. Sterols were extracted from 150 mg ground fresh weight with 4 ml dichloromethane: methanol (2:1, V/V) containing 60 µg ergosterol as internal standard. After the organic phases were dried and redisolved in 100 µl tetrahydrofuran and 100 µl N-methyl-N-(trimethylsilyl) trifluoroacetamide, 1 µl of the extract was injected into a GC-MS instrument for analysis. Bars represent mean ± SE of 3 replicates. Different letters on each bar represent significant differences after ANOVA ($F_{3,8} = 13.69$, $p < 0.01$) followed by Tukey HSD post hoc test at $\alpha = 0.05$.

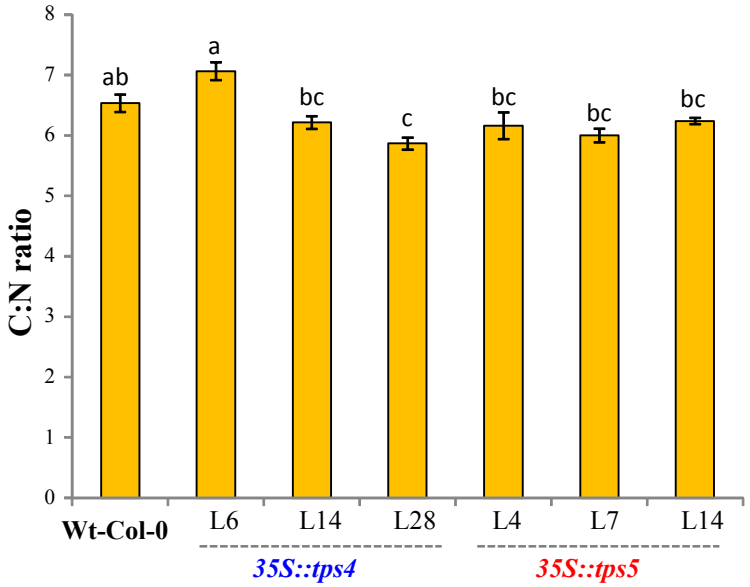

Fig. S9.1.5: Comparison of C:N ratios of wild-type and transgenic plants. Bars represent mean ± SE of 4 to 32 replicates. Different letters on each bar represent significant differences after ANOVA ($F_{6,72} = 206.46$, $p < 0.001$) followed by Tukey HSD post hoc test at $\alpha = 0.05$.

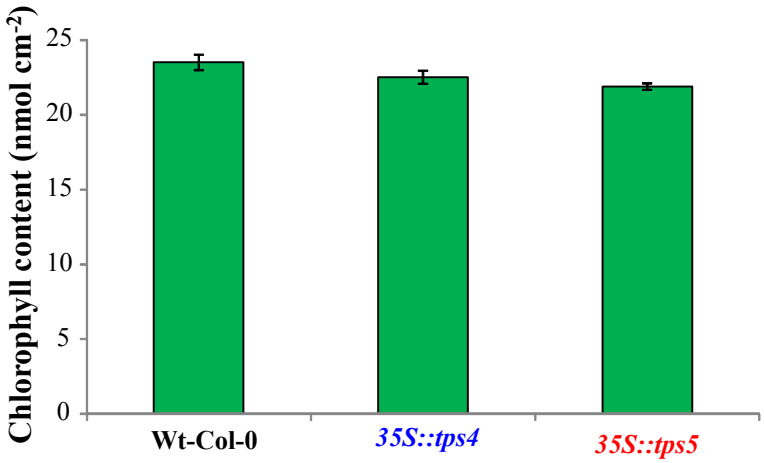

Fig. S9.1.6: Comparison of chlorophyll contents of wild-type and transgenic plants. Bars represent mean ± SE of 16 to 24 random measurements with a SPAD 502 Plus Chlorophyll Meter (Spectrum Technologies, Inc.) and converted to nmol per unit area using Ling et al., 2011 formula derived for Arabidopsis plants.

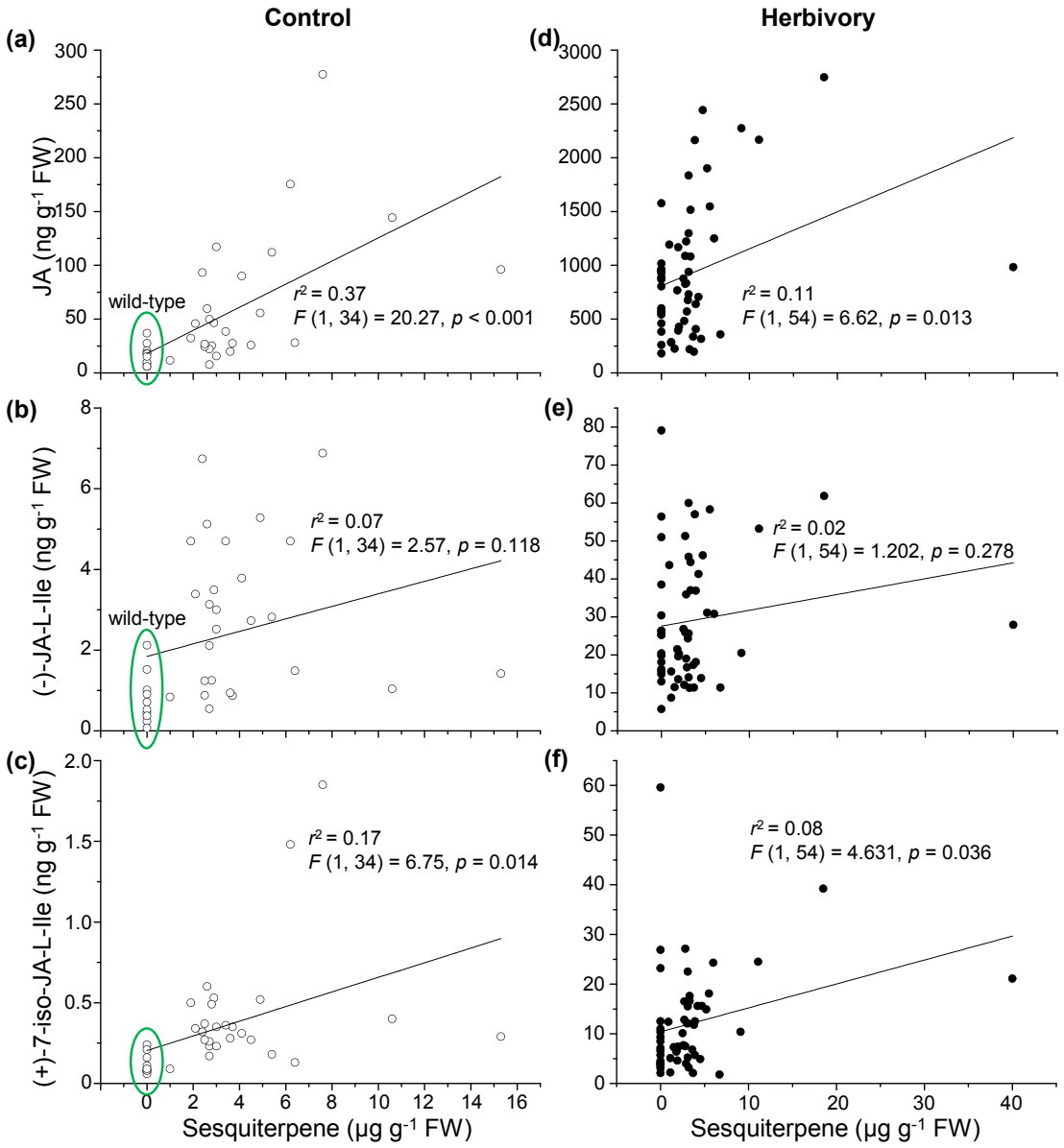

Fig. S9.1.7: Relationship between jasmonate and sesquiterpene concentrations in the transgenic lines prior to **(Control)** or after 24 h *S. littoralis* herbivory **(Herbivory)** treatment.

9.2 Supplementary material for Research Chapter II

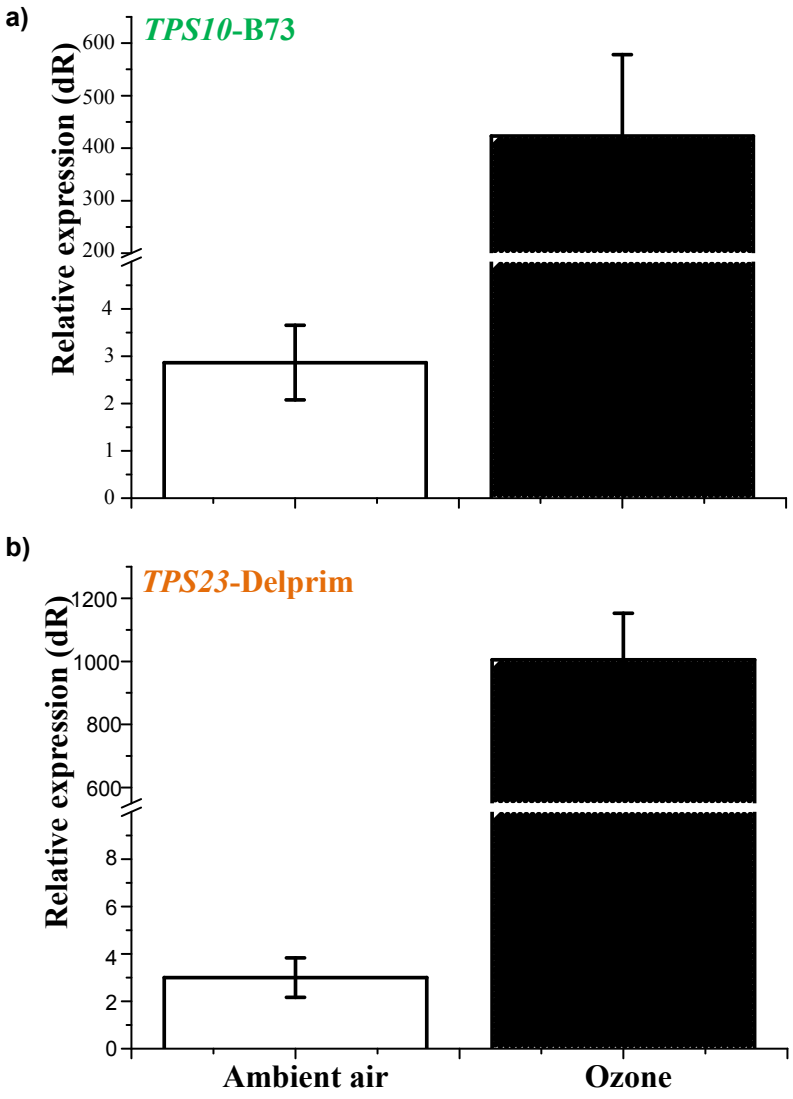

Fig. S9.2.1: Relative gene expression analysis of the maize t*ps10* and *tps23* genes after acute ozone (300 ppb) exposure for 6 h. Primer pairs used to probe the expression of the genes are listed in the primers list table under Table S9.2.a.

Table S9.2.1: Primer pairs used for the gene expression analysis

Gene name	Application	Primer sequences 5'- 3'	Size (bp)
Zmtps10-B73	ORF amplification	Fwd: ATGGATGCCACCGCCTTC Rev: CTAGAATAATGATATTGGAT	1602
Zmtps23-Delprim1	ORF amplification	Fwd: ATGGCAGCTGATGAGGCAAGATCCG Rev: TTAGTCTATTAGAT GCACATACAATG	1644
Zmtps10-B73	qRT-PCR probe	Fwd: AGGGAACTTCGTGGTGGATGATAC Rev: TGGCGTCTGGTGAAGGTAATGG	114
Zmtps23-Delprim1	qRT-PCR probe	Fwd: TCTGGATGATGGGAGTCTTCTTTG Rev: GCGTTGCCTTCCTCTGTGG	130
AtMPK3	qRT-PCR probe	Fwd: TGACGTTTGACCCCAACAGA Rev: CTGTTCCTCATCCAGAGGCTG	146
AtCAT1	qRT-PCR probe	Fwd: AGGAGCCAATCACAGCC Rev: TCAAGACCAAGCGACCA	194
AtACS6	qRT-PCR probe	Fwd: TCCCGGCGATGGTTTCTTAGTTC Rev: TCCAAGGCTTCCACCGTAATCTTG	138
AtABI1	qRT-PCR probe	Fwd: CCGTTGTTTTCCCGTCTCA Rev: CGGCAAAGAACGGCTCTAGA	64
AtActin8	Normalizer	Fwd: ATGAAGATTAAGGTCGTGGCAC Rev: GTTTTTATCCGAGTTTGAAGAGGC	400

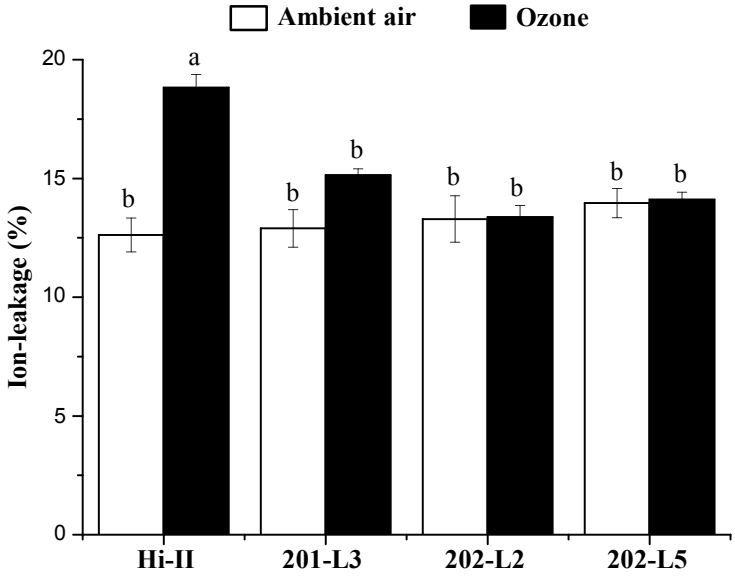

Fig. S9.2.2: Ozone-induced ion leakages of wild-type and transgenic Hi-II maize lines. Leaf samples were collected (14 mm Ø) after the plants were exposed to either ambient air or 300 ppb ozone for 6 h. Samples were floated on deionized water for 3 h and the conductivity of the water was measured. Ion-leakage was expressed relative to the conductivity recorded after the samples were boiled to get the total electrolyte. The graph shows mean ± SE of 5 replicates. Bars with different letters represent significant differences at $\alpha = 0.05$ after ANOVA followed by Tukey HSD analysis.

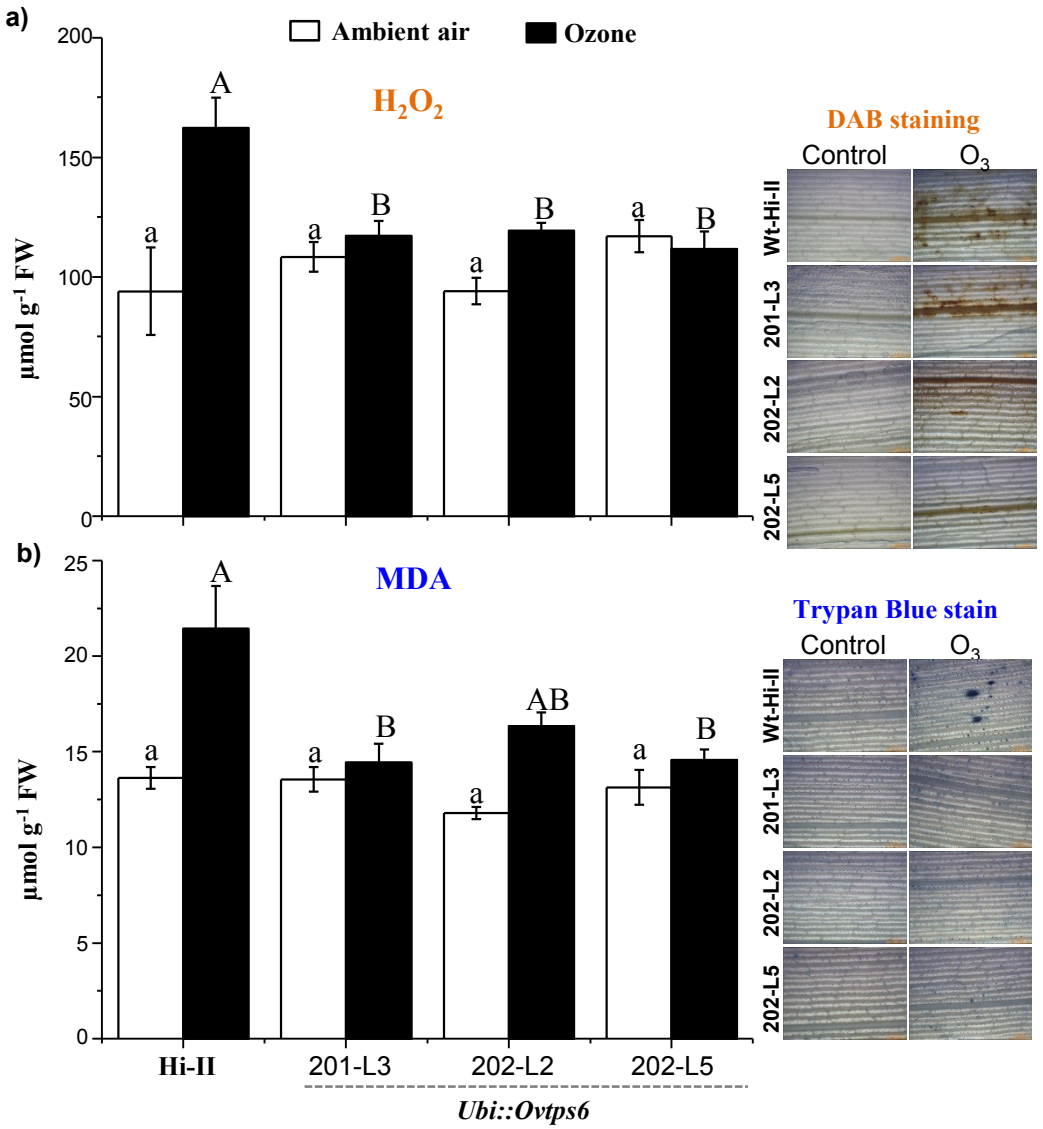

Fig. S9.2.3: Quantification of ozone-induced hydrogen peroxide (H_2O_2) and malonyldialdehyde (MDA) accumulation. Seedlings (10 days-old) were exposed to ozone (300 ppb, 6 h) and third and fourth leaves were pooled for the extraction of **(a)** H_2O_2 and **(b)** MDA. The third leaf was also histochemically stained with DAB (3,3'-diaminobenzidine) and trypan blue to qualitatively analyse H_2O_2 and cell death respectively. Deep brown coloration represents H_2O_2 accumulation and blue stains represent cell deaths. Bars with different letters represent significant differences at $\alpha = 0.05$, n = 5 replicates after ANOVA followed by Tukey HSD analysis.

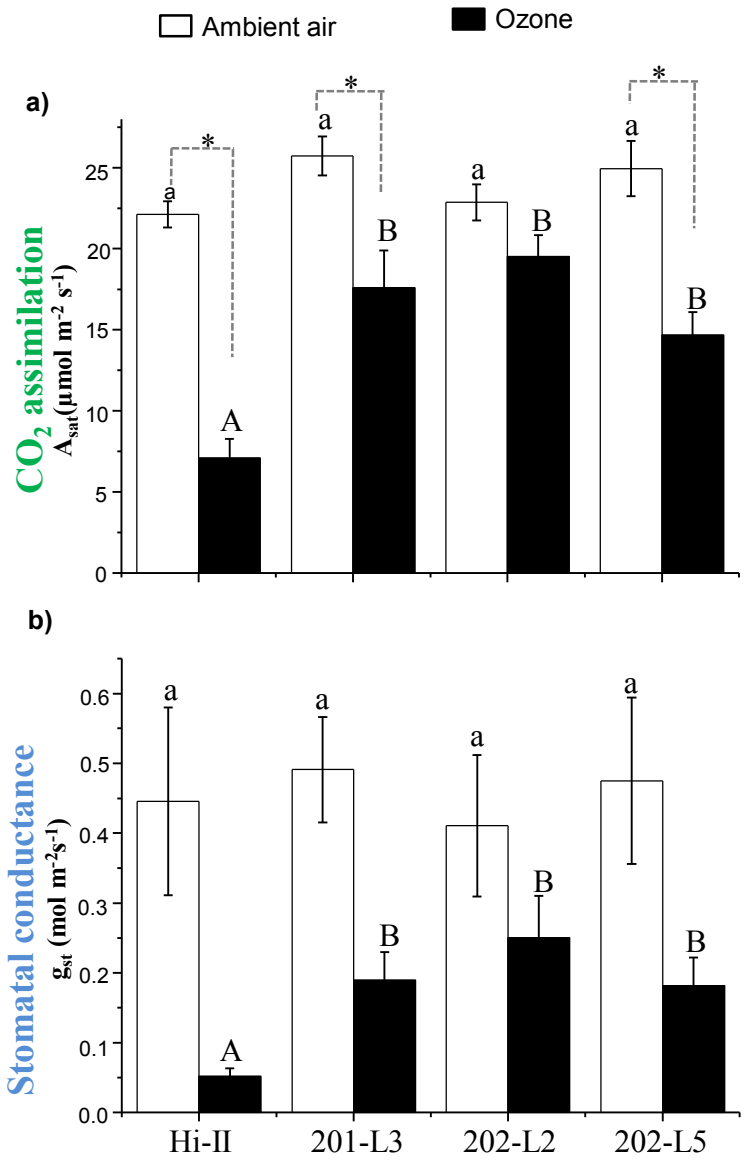

Fig. S9.2.4: Infrared gas exchange analysis on ozone-exposed maize leaves. Wild-type and *(E)*-β-caryophyllene overproducing transgenic Hi-II maize lines were exposed to either ambient air or ozone (300 ppb, 6 h). After 18 h recovery, CO_2 assimilation rate (A_{sat}) **(a)** and stomatal conductance (g_{st}) **(b)** were measured. Gas analysis was conducted at 400 µmol mol^{-1} CO_2 concentration and a saturated photo flux (Q) of 1500 µmol m^{-2} s^{-1}.

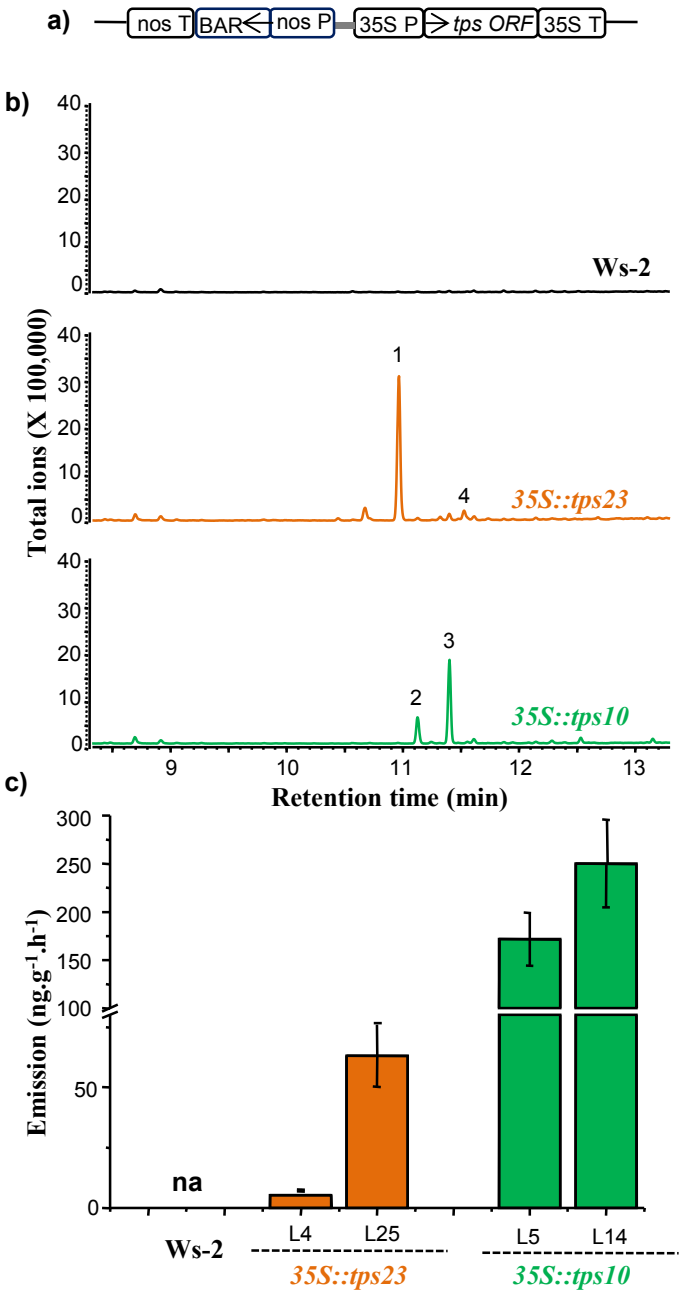

Fig. S9.2.5: Engineering of transgenic Arabidopsis plants that express maize sesquiterpene synthase genes. **(a)** *35S::tps* construct scheme used to transform *Arabidopsis thaliana*, ecotype Ws-2. **(b)** Headspace volatile collection from four week-old rosette leaves of Arabidopsis and subsequent analysis with GC-MS. The sesquiterpenes identified in the transgenic lines are as follows: 1. *(E)*-β-caryophyllene; 2. *(E)*-α-bergamotene; 3. *(E)*-β-farnesene; and 4. *α*-humulene. **(c)** Quantification of the products identified with GC-FID.

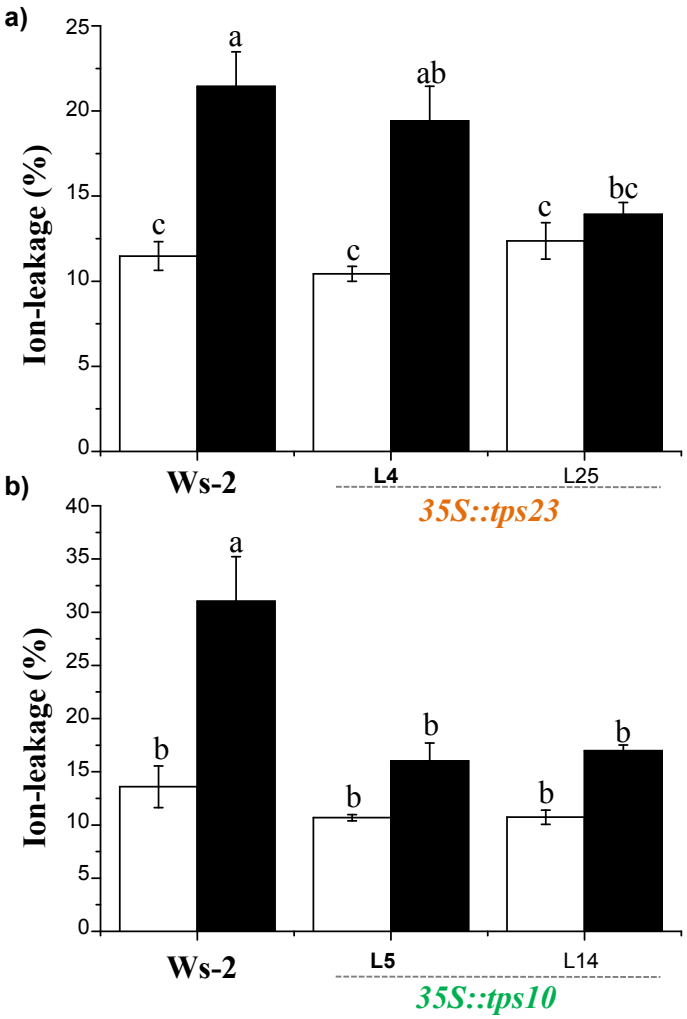

Fig. S9.2.6: Ozone-induced ion leakages of wild-type and sesquiterpene over- expressing transgenic Ws-2 lines. Leaf samples were collected (14 mm Ø) after the plants were exposed to either ambient air or 300 ppb ozone for 6 h. Samples were floated on deionized water for 3 h and the conductivity of the water was measured. Ion-leakage was expressed relative to the conductivity recorded after the samples were boiled to get the total electrolyte. The graph shows mean ± SE of 5 replicates. Bars with different letters represent significant differences at $\alpha = 0.05$ after ANOVA followed by Tukey HSD analysis.

9.3 Supplementary material for Research Chapter III

Table S9.3.1: Primer pairs used for the gene expression analysis

Gene name	Application	Primer sequences 5'- 3'	Size
Cg*Actin*	Fungal infection assessment	Fwd:TTCCGTCCTTGGTCTTGAG Rev:GTGTTAGGTGATGAATCTTCTTAG	154
Cgβ-tubulinA	Fungal infection assessment	Fwd:GTGACGGACGCTTCTTGAC Rev: GCTGAGTTCTTGTTCTTGATGG	100
ZmPR10	qRT-PCR probe	Fwd:GGCGTGGAGGTGAAGGAC Rev: GGTTGGCGACGAGGTAGG	88
ZmBx7	qRT-PCR probe	Fwd:CGAAGGAGAACACTACTACC Rev: TGAACAGCAGGGAGATGG	79
ZmActin1	qRT-PCR probe	Fwd:CTCACCGACCACCTAATG Rev: CATCAGGCATCTCGTAGC	181
ZmRPB1	Normalizer	Fwd:GCTGGATGATGAGAATTGGAGACC Rev:GCTTGAGGTTCACAGGCATAGG	188

10. Acknowledgement

YOU know LORD, how you raised me up from scratch! When I see back the entire journey that I have been through starting from my childhood, I have no words to thank GOD for his blessing and protection to have all the endurance and the patience to reach at this level.
I would like to thank the following people and institutions:

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- Angela Schneider for arranging all the official proceedures for my stay in Jena.
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- Dr. Tobias Köllner for his helpful discussions on my projects and manuscripts.
- Dr. Lawrence Wright for introducing me the PTR-MS and Infrared gas exchange analytical techniques and for his valuable suggestions on my projects.
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- Dr. Almuth Hamerbacher for her technical advice on antifungal bioassays and statistical analysis.
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- Daniel Rothenberger for his technical support on insect feeding bioassays.
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11. Abridged Curriculum Vitae

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Date	**01.07.2002 – 30.08.2004**
Position held	Graduate Assistant at Hawassa University, Awassa, Ethiopia
Responsibilities	Teaching and research in the Department of Plant Science
Education & Research	
Date	**01.01.2007-02.10.2014**
Title of qualification awarded/expected	Scientific co-worker and Doctoral candidate at the Max-Planck Institute for Chemical Ecology, Department of Biochemistry, Hans-Knöll-Str.8, 07745 Jena, Germany
Doctoral thesis	Investigating the roles of volatile sesquiterpenes in plant defence against biotic and abiotic stresses
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Master thesis	Testing the transcriptional regulation of *Arabidopsis thaliana* PhospholipaseA gene families using natural and synthetic promoters
Date	**01.10.1998 – 30.07.2002**
Qualification awarded	BSc. in Plant science, Hawassa University, Awassa, Ethiopia
Principal subjects	Plant breeding, Plant protection, Soil science, Forestry, Plant physiology, etc
Further training	
Date	**16.03.2008 – 20.03.2008**
Qualification awarded	Certificate in Bioinformatics, European Genetics Foundation, Bologna, Italy
Date	**09.10.2002 – 20.10.2002**
Qualification awarded	Certificate in integrated and participatory approaches in agrobiodiversity, plant breeding and seed production, International Agriculture Centre (IAC) Wageningen University

Awards	**Gold medal** given to one outstanding student in the 2002 BSc graduating class of Hawassa University
	DAAD scholarship to pursue my MSc degree at the University of Hannover (**2004 –2006**)
Language	Amharic: Mother tongue, English: Proficient user, German: Basic user

Publications:

Fontana A, Held M, **Fantaye CA**, Turlings TC, Degenhardt J, Gershenzon J (2011). Attractiveness of constitutive and herbivore-induced sesquiterpene blends of maize to the parasitic wasp *Cotesia marginiventris* (Cresson). *Journal of Chemical Ecology* **37:** 582-591

Fantaye CA, Degenhardt J, Gershenzon J (2014). Plant volatiles as key players in diverse ecological interactions. *Journal of Endocytobiosis and Cell Research*: **25:** 1-8

Fantaye CA, Köllner T, Gershenzon J, Degenhardt J. Volatiel sesquiterpenes prime herbivore induction of plant defenses in genetically engineered *Arabidopsis thaliana*. This manuscript is submitted (2014) to the *Journal of New Phytologist*

Fantaye CA, Köpke D, Gershenzon J, Degenhardt J. Genetically restoring *(E)*-β-caryophyllene production in a non-producing maize line compromises its resistance against the fungus *Colletotrichum graminicola.* This manuscript is submitted (2014) to the *Journal of Chemical Ecology*

Fantaye CA, Gershenzon J, Degenhardt J. Transgenic Arabidopsis plants emitting *(E)*-β-caryophyllene or *(E)*-β-farnesene type of sesquiterpene are better protected from ozone-induced injury. In preparation for publication

Oral and poster presentations:

Fantaye CA, Gershenzon J, Degenhardt J. Functional characterization of maize terpenes by expressing biosynthetic genes in Arabidopsis. 6th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany, Mar 2007

Fantaye CA, Gershenzon J, Degenhardt J. Expression of maize terpene synthases in *Arabidopsis thaliana* to study their regulatory mechanisms and roles in plant defense. ICE Symposium, MPI for Chemical Ecology, Jena, Germany, Sep 2007

Fantaye CA, Gershenzon J, Degenhardt J. Functional characterization of sesquiterpenes by expressing biosynthetic genes in Arabidopsis. 7th Biannual IMPRS symposium, Dornburg, Germany, Feb 2008

Fantaye CA, Gershenzon J, Degenhardt J. Ozone-induced cell death: Do sesquiterpenes influence the signaling process? ICE symposium, MPI for Chemical Ecology, Jena, Germany, Jun 2009

Fantaye CA, Gershenzon J, Degenhardt J. The role of maize volatile sesquiterpenes in direct defense against herbivores. 8th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany, Mar 2009

Jena, October 2, 2014 Chalie Assefa Fantaye

12. Eigenständigkeitserklärung

Die zurzeit gültige Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena ist mir bekannt. Die vorliegende Arbeit wurde von mir selbst und nur unter Verwendung der angegebenen Hilfsmittel erstellt und all benutzen Quellen angegeben. All Personen, die an der experimentellen Durchführung, Auswertung des Datenmaterials oder bei der Verfassung der Manuskripte beteiligt waren, sind benannt. Es wurde weder bezahlte noch unbezahlte Hilfe eines Promotionsberaters in Anspruch genommen. Die vorliegende Arbeit wurde bisher weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch bei einer anderen Hochschule als Dissertation eingereicht.

Jena, den 28.04.2014 Chalie Assefa Fantaye